

**STRUCTURE AND FUNCTION OF THE BOVINE CHROMAFFIN GRANULE PROTON PUMP**

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***A Isabel***

## DECLARATION

The work presented in this thesis is my own and was carried out while a member of a research group. Some of the results presented herein have already been published. The work was directed and supervised by Dr. D.K. Apps.

The thesis presented here has been composed by myself.

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## ABSTRACT

The secretory granules of the adrenal medulla, known as "chromaffin granules", store catecholamines, proteins and nucleotides that afterwards will be secreted by exocytosis. The accumulation of catecholamines within the granules occurs by a chemiosmotic mechanism involving a membrane-bound  $H^+$ -translocating ATPase, coupled to a separate  $H^+$ -catecholamine exchanger. This ATPase belongs to a recently identified group of proteins called "vacuolar" or V-type ATPases, that are distinct from the P-type ATPases of fungal plasma membranes and F-type  $H^+$ -ATPases of energy transducing membranes.

The aim of this thesis is the structural and functional characterization of the bovine chromaffin granule ATPase. First, a new and rapid method of purification and reconstitution of the enzyme was developed. The method can be accomplished in less than two hours, yielding a partially purified form of the enzyme incorporated into liposomes of defined composition. This permitted the measurement of the  $H^+$ -translocation activity of the enzyme by use of the fluorescent probe ACMA. The effect of lipid composition on ATP hydrolysis and  $H^+$ -translocation activities was studied.

Kinetic and regulatory properties were carried out on the reconstituted enzyme. MgATP, MgADP, MgGTP and MgITP were all shown to be substrates for the enzyme and  $Mn^{2+}$ ,  $Ca^{2+}$ ,  $Co^{2+}$  and  $Ni^{2+}$  could substitute for  $Mg^{2+}$ . Kinetic parameters were calculated for all these cases. The inhibitory effects of ADP, GDP and IDP were also studied. The regulatory properties were consistent with the existence of several substrate and inhibitor binding sites and the kinetic data could be fitted to a model involving positive homotropic and negative heterotropic effects.

Structural studies of the purified enzyme were performed. It was shown that one of the subunits (120 kDa) was a glycoprotein. A hydrophobic probe specifically labelled two subunits (120 and 16 kDa) suggesting that they form the part of the enzymic complex embedded in the granule membrane. The stoichiometry of the complex was calculated from quantitative amino acid analysis of the subunits separated by electrophoresis in polyacrylamide gels. In order to do this a purer form of the enzyme was obtained by centrifugation through glycerol density gradients. This separated the active enzyme from a complex formed by polypeptides of molecular masses 116, 40, 19 and 16 kDa, suggesting that the enzyme contains two separable domains.

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## ABBREVIATIONS

ACMA: 9-amino-6-chloro-2-methoxyacridine  
ATP: adenosine-5'-triphosphate  
ATPase: adenosine-5'-triphosphatase (EC 3.6.1.3.)  
BACH: biotinamidocaproilhydrazide  
BSA: bovine serum albumin  
C<sub>12</sub>E<sub>9</sub>: isotridecylpoly(ethyleneglycolether)<sub>8</sub>  
CTP: cytosine-5'-triphosphate  
 $\Delta\mu\text{H}^+$ : Proton electrochemical gradient  
 $\Delta\text{pH}$ : Transmembrane pH gradient  
 $\Delta\psi$ : Transmembrane potential  
DBH: dopamine- $\beta$ -monooxygenase  
DCCD: N,N'-dicyclohexylcarbodiimide  
DMSO: dimethylsulphoxide  
DTT: dithiothreitol  
EDTA: ethylenediaminetetraacetic acid  
FCCP: carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone  
GTP: guanosine-5'-triphosphate  
HEPES: N-(2-hydroxyethyl)piperazine-N'-2-ethanesulphonic acid  
ITP: inosine-5'-triphosphate  
kDa: Kilodalton  
 $\mu\text{Ci}$ : microcurie  
MBq: Megabecquerel  
MEGA-8: N-(D-gluc-2,3,4,5,6-pentahydroxyhexyl)-N-methyloctanamide  
MES: 2-(N-morpholino)ethanesulphonic acid  
Nbf-Cl: 4-chloro-7-nitrobenzofurazan  
NEM: N-ethylmaleimide  
pI: Isoelectric point  
PC: phosphatidylcholine  
PE: phosphatidylethanolamine  
PMSF: phenylmethylsulphonyl fluoride  
PS: phosphatidylserine  
r.p.m.: Revolutions per minute  
SDS: sodium dodecyl sulphate  
SDS-PAGE: polyacrylamide gel electrophoresis of proteins in sodium dodecyl sulphate

S-SHPP: sulfosuccinimidyl-3-(4-hydroxyphenyl)propionate

TBq: Terabecquerel

TEMED: N,N,N',N'-tetramethylethylenediamine

TLCK: N $\alpha$ -p-tosyl-L-lysine chloromethyl ketone

TPCK: N-tosyl-L-phenylalanine chloromethylketone

TRIS: tris(hydroxymethyl)aminomethane

UTP: uridine-5'-triphosphate



**Chapter 1**  
**INTRODUCTION**

## **1.1 BIOLOGY OF THE CHROMAFFIN CELL**

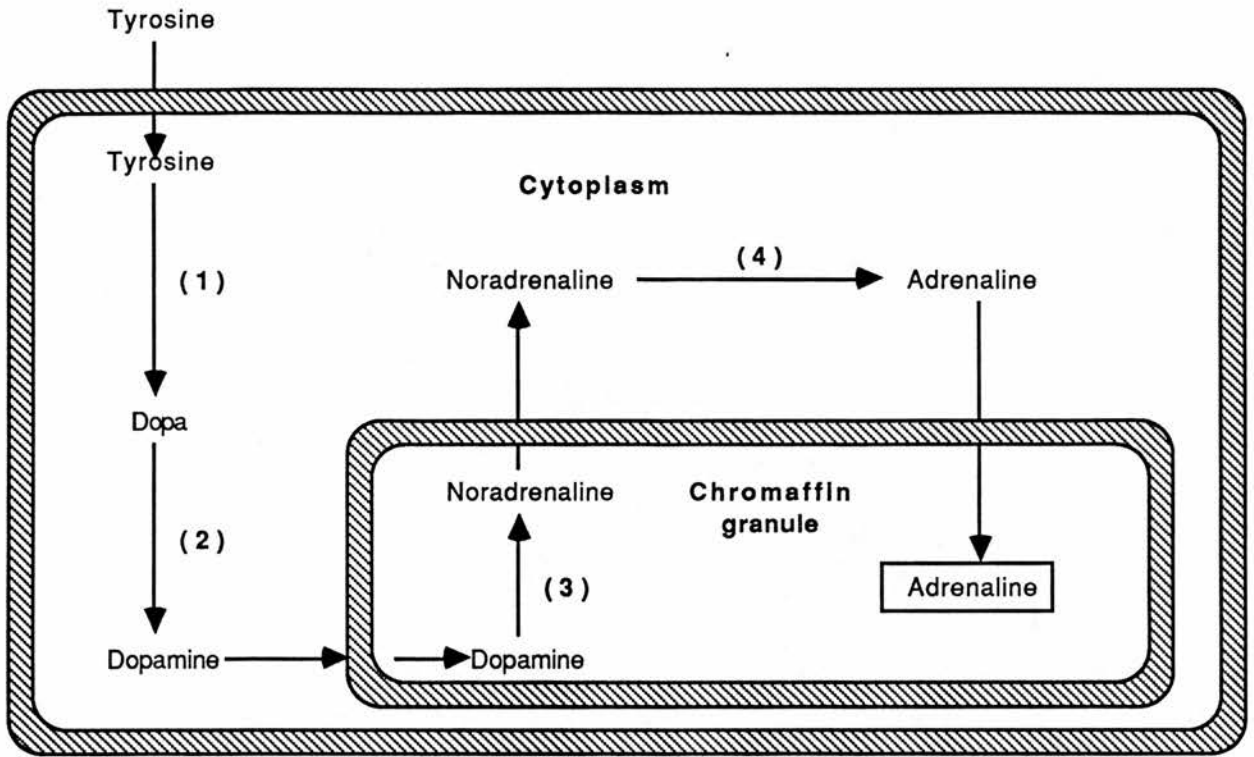
The mammalian adrenal gland is composed of two distinct tissues, the medulla and the cortex. The reddish-brown cortex surrounds the pink medulla and is involved in synthesis and secretion of steroid hormones (glucocorticoids and mineralocorticoids). Embryologically, the cortex is of mesodermal origin whereas the medulla is of ectodermal. The cells of both tissues develop from the neural crest.

The adrenal medulla is involved in the synthesis, storage and secretion of catecholamines; various neuropeptides and a group of acidic proteins (the chromogranins, of unknown function) are also secreted in the same process. The adrenal medulla is composed of a type of cells related to the adrenergic nervous system, called "chromaffin cells". This name was given by Alfred Kohn when he observed that, by treatment of the cells with chromium salts, a yellowish-brown colour developed. The majority of chromaffin cells synthesize and store adrenaline and a small number store noradrenaline. However, the proportion of each cell type varies from one species to another. Catecholamines are stored within the cell in a large number of small membrane-enclosed vesicles called "chromaffin granules".

### **1.1.1. Biosynthesis of catecholamines.**

Within the chromaffin cell, a number of biosynthetic steps occur in the production of catecholamines from the precursor aminoacid, tyrosine. Tyrosine is hydroxylated by the cytoplasmic enzyme tyrosine 3-hydroxylase (EC.1.14.16.2) to form 3,4-dihydroxyphenylalanine (dopa), which is subsequently decarboxylated to form dopamine by dopa decarboxylase (EC.4.1.1.28). Dopamine is then taken into the chromaffin granule where it is hydroxylated by dopamine- $\beta$ -monooxygenase, more often known as dopamine- $\beta$ -hydroxylase or DBH (EC.1.14.17.1), an exclusively intragranular enzyme. In noradrenaline-containing chromaffin cells the pathway stops here, but in the adrenaline containing ones, noradrenaline re-enters the cytoplasm where it is converted to adrenaline by phenylethanolamine N-methyltransferase (EC.2.1.1.28), found only in adrenaline-containing cells. (Figure 1.1).

There are between 20,000 and 30,000 chromaffin granules in each chromaffin cell (Phillips, 1982) and their role in catecholamine secretion can be summarized as follows: (1) biosynthesis of adrenaline, since DBH is only present within the granule; (2) concentration and packaging of catecholamines against a huge concentration gradient, allowing a large amount of these to be released by exocytosis; (3) maintenance of the catecholamines in an acidic pH and in the presence of ascorbate to prevent their oxidation; (4) release of catecholamines by regulated exocytosis. As noted below, chromaffin granules also store and secrete a variety of neuropeptides.



**Figure 1.1. Biosynthesis of catecholamines within chromaffin cells.** Numbers in brackets indicate the enzyme involved in the step: (1) Tyrosine-3-hydroxylase; (2) dopa decarboxylase; (3) dopamine- $\beta$ -monooxygenase; (4) phenylethanolamine-N-methyl transferase.

### 1.1.2. The mechanism of secretion.

The major physiological stimulus for exocytosis in chromaffin cells is neurally released acetylcholine acting on the chromaffin cell nicotinic receptors. This causes the opening of the receptor channel allowing the entry of  $\text{Na}^+$ . The membrane polarization thus obtained activates voltage sensitive ion channels allowing a further entry of  $\text{Na}^+$  and  $\text{Ca}^{2+}$ . The overall effect is that the cytosolic free  $\text{Ca}^{2+}$  concentration increases from about  $0.1 \mu\text{M}$  before stimulation to  $1 \mu\text{M}$ . This increase is the primary trigger for exocytosis provoking the movement of chromaffin granules towards the plasma membrane where both membranes fuse and the granule content is released into the bloodstream (Burgoyne, 1984).

A subsidiary role of chromaffin granules may be the control of the cytosolic concentration of  $\text{Ca}^{2+}$  after exocytosis, by uptaking about 10% of the calcium that enters the cell (Haigh and Phillips, 1989). The remaining 90% is presumably extruded by the plasma membrane  $\text{Ca}^{2+}$ -ATPase and/or  $\text{Ca}^{2+}/\text{Na}^+$  exchanger.

The precise mechanism that transduces the cytosolic  $\text{Ca}^{2+}$  signal into the mechanics of granule and plasma membrane fusion is not clearly understood. It has been suggested that, in unstimulated cells, exocytosis is prevented by the immobilization of the granules in a cytoskeletal network, the  $\text{Ca}^{2+}$  entry causing the disruption of this network and allowing the granules to move towards the plasma membrane and fuse with it (Burgoyne, 1984). However, several important aspects of exocytosis remain unsolved such as the effect of G-proteins on the cytoskeleton, the nature of events involved in the membrane fusion and the precise role of MgATP, shown to be essential for exocytosis by several authors (Knight and Baker, 1982).

### 1.1.3. Reasons for the study of chromaffin granules.

Because the cells of the adrenal medulla and the sympathetic nervous system have a common embryological origin, it is often assumed that they have similar mechanisms for the synthesis, storage and release of catecholamines and neurotransmitters. Therefore, studies of these phenomena in the secretory granules of chromaffin cells may be extrapolated to other secretory organelles. Indeed, results obtained in studies with other amine-containing granules, such as the 5-hydroxytryptamine-containing granules of platelets, histamine granules of mast cells and glutamate-secretory vesicles from brain (Maycox *et al.*, 1988) are in many ways similar to those obtained with chromaffin granules. Even acetylcholine-storing granules from cholinergic neurons and insulin-storage granules of the pancreas show some points of comparison.

The main advantages of the chromaffin granule as a model neuroendocrine secretory granule can be listed as follows: (a) adrenal glands are easily obtained in large amounts from slaughterhouses, and each medulla yields a huge amount of granules; (b) chromaffin granules are easy to isolate and to separate from contaminating subcellular organelles such as mitochondria by density gradient centrifugation, due to their high buoyant density; (c) purified chromaffin

granules can be subjected to hypoosmotic shock to release their contents, and resealed to form "ghosts", which lack endogenous ion gradients and intragranular matrix contents but which can accumulate catecholamines (Phillips, 1974a). This allows one to vary the internal matrix composition at will, and concentration gradients achieved during catecholamine uptake can easily be calculated; (d) the granule membranes themselves are easily purified, and yields of 50-100 mg of membranes are readily achieved from about 50 glands.

#### **1.1.4. Composition of chromaffin granules.**

Most studies have been carried out with bovine adrenal glands (see Table 1.1); the composition of the granules varies somewhat between species.

##### **1.1.4.1. Granule matrix.**

The major components of the chromaffin granule matrix are, obviously, catecholamines, whose internal concentration has been calculated to be around 550 mM (Winkler, 1976). Nucleotides are also present in a high concentration (about 180 mM, ATP being 70% of the total). Their function is unknown, although they have been proposed to act by decreasing the osmotic pressure generated by the catecholamines (Winkler and Westhead, 1980). Other components are ascorbate (23 mM), the electron donor in the reduction of dopamine to noradrenaline (see below), and cations such as  $\text{Ca}^{2+}$  (20 mM),  $\text{Mg}^{2+}$  (5 mM) and  $\text{Na}^+$  (22 mM).

The total protein content of the granule is about 180 mg/ml, most of it being a family of acidic proteins originally called chromogranins some of which are now called secretogranins: Chromogranin A is the major one in bovine tissue, making up 50% of the total matrix content; it is very acidic (pI: 4.8-5.6) and is O-glycosylated with galactose, N-acetylglucosamine and sialic acid as the major sugars. This protein has been cloned and sequenced (Iacangelo *et al.*, 1986; Benedum *et al.*, 1986) and shown to consist of 431 aminoacids with a high content of glutamic acid and proline. Its molecular mass is 48 kDa compared with its apparent molecular mass of 70-77 kDa from SDS-PAGE. Human chromogranin B (secretogranin I) has also been sequenced (Benedum *et al.*, 1987) and shown to have some homology with chromogranin A and a similar pI, although it has a molecular mass of 76 kDa. Chromogranin C (secretogranin II) has a molecular mass of 86 kDa and a pI of about 5. Each of these chromogranins undergoes post-translational proteolysis, so that the mature granule contains an assortment of chromogranin-derived proteins, some of very low molecular mass.

A wide occurrence of the three types of chromogranins has now been reported in numerous endocrine and neural tissues (Winkler *et al.*, 1986) although their function is still unknown. However, some possible functions have been suggested; thus, chromogranin A (and possibly B and C) binds much of the 20 mM  $\text{Ca}^{2+}$  present in the matrix (Reiffen and Gratzl, 1986) and it has

been postulated to be a sorting element involved in segregation of proteins into the secretory pathway (Benedum *et al.*, 1986). NMR studies have also shown that chromogranin A appears to be random coil and in rapid motion, which suggests that it does not contribute greatly to catecholamine binding within the matrix (Winkler and Westhead, 1980). The sequence of chromogranin A contains that of pancreastatin which suggests that chromogranin-derived peptides may have a role after secretion (Huttner, 1991). A proteoglycan present in the granule matrix also seems to be a derivative of chromogranin A.

Apart from the chromogranins, various neuropeptides occur in the chromaffin granule matrix, including enkephalin precursors. Also present are a number of glycoproteins, many of still unknown function. Glycoprotein III is a disulfide-linked heterodimer with 37 kDa subunits, which also exists in a soluble form (Palmer and Christie, 1990). Another major glycoprotein of the granule matrix is a soluble form of dopamine- $\beta$ -monooxygenase, discussed below. Glycoproteins J and K (Gavine *et al.*, 1984) have been identified as carboxypeptidases, presumably involved in protein processing (Hook *et al.*, 1985). Enkephalins are also produced in the granule matrix by degradation of proenkephalin A by proteases giving Met- and Leu-enkephalins (Winkler *et al.*, 1986). Finally, a host of small peptides are also present: neuropeptide Y, bombesin, vasoactive intestinal peptide and dynorphin.

#### 1.1.4.2. Granule membrane.

The chromaffin granule membrane is highly impermeable to cations in general (see below) and is characterized by its high lipid content (2  $\mu\text{mol}$  of phospholipid per mg of protein). Within the lipids there is a high content of cholesterol (0.6  $\mu\text{mol}/\mu\text{mol}$  of phospholipid) and lysophosphatidylcholine (0.18  $\mu\text{mol}/\mu\text{mol}$ ). This latter is asymmetrically distributed with only 10% in the outer face of the membrane (Winkler and Westhead, 1980). As far as proteins are concerned, one-dimensional SDS-PAGE reveals at least 40 bands (Abbs and Phillips, 1980). Amongst these, membrane-associated DBH accounts for 25-30% of the total membrane protein content. This enzyme is involved in the reduction of dopamine to noradrenaline (see above) and it has been shown to contain four subunits of 70-75 kDa which are probably not identical. It is a copper-containing glycoprotein (4% sugar) with N-linked polysaccharides, and is located entirely on the inner side of the membrane. The membrane-bound DBH only differs from the soluble form in having an extra hydrophobic domain that attaches it to the membrane (Winkler *et al.*, 1986), but the nature of this hydrophobic anchor is still unknown. By transfer to nitrocellulose of SDS-PAGE analysed membranes and decoration with various lectins, about 20 glycoproteins have also been identified (Gavine *et al.*, 1984; Pryde and Phillips, 1986). Most studies indicate that the sugar residues of these proteins are on the luminal side of the granule membrane. The function of most of these proteins is unknown. The properties and structure of cytochrome  $b_{561}$  and  $\text{H}^+$ -ATPase I, also membrane-bound proteins, will be discussed below.



**Table 1.1. Composition of Bovine Chromaffin Granules.**

**Matrix Proteins and Peptides.**

Chromogranin A family	}	approx. 180 mg/ml (Chromogranin A about 50% of total; DBH about 4%)
Chromogranin B (secretogranin I) family		
Chromogranin C (secretogranin II) family		
Proenkephalin family		
Acetylcholinesterase		
Proteases		
Dopamine- $\beta$ -hydroxylase		

<b>Matrix glycosaminoglycans</b>	<b>nmol sugar/mg membrane protein</b>
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Chondroitin sulphate	2600
Heparin sulphate	100

<b>Matrix small molecules</b>	<b>nmol/mg granule protein</b>	<b>mM</b>
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Adrenaline	1800	
Noradrenaline	680	580
Dopamine	20	
ATP	560	130
GTP	76	18
UTP	43	10
ADP	66	15
AMP	38	9
Ascorbic acid	100	23
Enkephalins	7	1.6
Ca <sup>2+</sup>	90	20
Mg <sup>2+</sup>	20	5
Na <sup>+</sup>	94	22

<b>Membrane proteins</b>	<b>approx. Mol. Mass (kDa)</b>
Mg <sup>2+</sup> -ATPase I (proton pump)	400-600
Mg <sup>2+</sup> -ATPase II (unknown function)	140
Cytochrome b <sub>561</sub>	30
Dopamine-β-hydroxylase	290
Phosphatidylinositol kinase	?
Catecholamine transporter	?
Ca <sup>2+</sup> /Na <sup>+</sup> Exchanger	?
Glycoproteins (II, III, J, K)	(100, 37, 56, 53)
Synaptin	?
Synaptophysin	38

<b>Membrane lipids</b>	<b>nmol/mg membrane protein</b>
Cholesterol	1440
Phosphatidylcholine	650
Lysophosphatidylcholine	410
Sphingomyelin	290
Phosphatidylethanolamine	840
Phosphatidylinositol	200
Phosphatidylserine	60
Ganglioside GM <sub>1</sub>	50
Fatty acids	100

<b>Glycosaminoglycans</b>	<b>nmol sugar/mg membrane protein</b>
Chondroitin sulphate	3000
Heparin sulphate	400

Data have been obtained or calculated from the following references: Gavine *et al.*, 1984; Geissler *et al.*, 1977; Phillips *et al.*, 1977; Ungar and Phillips, 1983; Winkler, 1976; Winkler and Westhead, 1980; Winkler *et al.*, 1986.



## 1.2. BIOENERGETICS OF THE CHROMAFFIN GRANULE.

The membranes of the secretory vesicles of the adrenal medulla, once regarded as little more than a wrapping for the cargo within, are in fact active structures exhibiting a variety of complex functions. These activities include transport, needed both to accumulate secreted substances and to expel unnecessary osmoticants, and electron transfer, needed to support intravesicular reactions.

As far as the function of transport is concerned, measurements of the internal water space (4.3  $\mu\text{l}/\text{mg}$  of protein: Phillips *et al.*, 1977) and catecholamine content (2500 nmol/mg protein) in isolated chromaffin granules led to the calculation of an apparent intragranular concentration of about 580 mM if all the catecholamines were free in solution (see Table 1.1). This is 4-5 orders of magnitude higher than the concentration in the surrounding cytosol. Obviously, in order to achieve this, a potent active transport is needed.

The elucidation of many of the predominant features of biogenic amine uptake was derived not from the direct study of amine transport, but rather from the investigation of the physicochemical properties of the chromaffin granule membrane.

### 1.2.1. Permeability of the chromaffin granule membrane.

Sensitive spectrophotometric and potentiometric techniques coupled with the appropriate use of ionophores or lipophilic membrane-permeable ions have permitted extensive investigation of the permeability of the chromaffin granule to small ions. From these studies, it was concluded that this membrane is highly impermeable to  $\text{H}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Na}^+$  and  $\text{K}^+$  (Johnson and Scarpa, 1976a). Moreover, the transmembrane  $\text{H}^+$  conductance is one order of magnitude less than across the inner mitochondrial membrane. The diffusion of anions across the membrane was higher than that for cations with relative permeabilities in the order of  $\text{SCN}^- > \text{I}^- > \text{Br}^- > \text{Cl}^-$ ,  $\text{SO}_4^{2-} > \text{F}^-$ , isothionate,  $\text{HPO}_4^{2-}$  (Phillips, 1977). These studies also indicated that, even when an ion is rendered freely permeable to the chromaffin granule membrane, there is no corresponding movement of a counterion (Johnson and Scarpa, 1976a).

All these physicochemical properties of the membrane may be related to the high content of lysophosphatidylcholine and cholesterol which creates a unique environment. From the physiological point of view, it makes sense that the properties of a subcellular organelle that contains such high amine concentrations should minimize any transmembrane ion fluxes that may affect the stability of the intragranular space and thus the distribution of the biogenic amines. The low proton permeability across the chromaffin granule is consistent with the idea of the existence of a pH gradient and a transmembrane potential.

### 1.2.2. The H<sup>+</sup>-translocating ATPase of the chromaffin granules and proton-linked transport of biogenic amines.

During the 1960s, Peter Mitchell elaborated his chemiosmotic theory that resolved the apparent paradox of how scalar energy forces could drive vectorial solute transport (Mitchell, 1976). The original chemiosmotic formulation is based in three basic principles: first, a topologically closed insulating membrane exists with a low permeability to ions and solutes; second, an anisotropically oriented H<sup>+</sup>-ATPase within the biological membrane generates an electrochemical proton gradient; third, proton-linked antiport or symport transporter within the membrane couple the electrochemical potential to ion or metabolic transport. As we have seen above, the chromaffin granule fulfills the first of these conditions and plenty of evidence has been raised in the last couple of decades to suggest that it also meets the other two requirements of the chemiosmotic theory.

The ATPase activity of chromaffin granules was first reported by Hillarp (1958) who also located this activity in the membrane by lysis of purified granules. In 1965, Banks reported the existence of a high intra-granular ATP concentration which is protected from hydrolysis; this fact further implied that the enzyme responsible for this activity resides on the outer surface of the granule.

Studies concerning the accumulation of catecholamines into chromaffin granules have shown that this accumulation can occur by means of two different mechanisms: one is an active transport that is ATP-dependent, reserpine- and NEM-sensitive (Kirshner, 1962) and also stereospecific for the R isomer (natural form); the other mechanism is a passive diffusion that lacks all these features (Slotkin and Kirshner, 1971).

The first evidence that the ATPase activity of the chromaffin granule could drive catecholamine uptake by creating a proton gradient across the membrane was obtained by Radda's group in a set of experiments in which they showed that, on addition of uncouplers believed to equilibrate protons across a membrane, the ATPase activity of chromaffin granule was enhanced and adrenaline incorporation suppressed (Bashford *et al.*, 1975a,b and 1976). Moreover, the specific transport inhibitor reserpine inhibited catecholamine uptake without affecting the ATPase activity. Further experiments produced the following observations: (1) isolated granules have an internal pH of about 5.5; this is slightly reduced on addition of ATP, although there is a much bigger change in the membrane potential (see below); (2) addition of ATP to isolated "ghosts" with the same internal pH as the medium resulted in a generation of a  $\Delta$ pH, acidic inside (Phillips and Allison, 1978); (3) a fixed stoichiometry of about 2H<sup>+</sup>/ATP existed between ATP hydrolysis and H<sup>+</sup>-translocation (Johnson *et al.*, 1982; Njus and Radda, 1978); (4) under appropriate conditions ATP could be synthesized at the expense of an imposed gradient (Roisin *et al.*, 1980).

The role of the chromaffin granule ATPase as a proton pump has been further supported by physicochemical studies carried out on the granule membrane. The difference between the

intragranular pH and the external pH ( $\Delta\text{pH}$ ) has been measured by different techniques, including [ $^{14}\text{C}$ ]methylamine distribution (Johnson and Scarpa, 1976*b*; Salama *et al.*, 1979), quenching of fluorescent amines (Bashford *et al.*, 1976) and [ $^{31}\text{P}$ ]NMR (Casey *et al.*, 1976 and 1977; Njus *et al.*, 1978; Pollard *et al.*, 1979). All these techniques agree that the internal pH is quite low, around 5.5-5.7, and is independent of the external pH and the ionic composition of the media.

The transmembrane potential ( $\Delta\psi$ ) can be measured from the distribution of radioisotopically labelled anions, and also spectrophotometrically (Pollard *et al.*, 1976; Salama *et al.*, 1979). When freshly isolated chromaffin granules are suspended in sucrose in the absence of anions, a resting potential of +10 to +20 mV is measured. Addition of MgATP produces a rapid and marked increase of  $\Delta\psi$  which reaches a plateau at -80 mV. The measurement of a  $\Delta\text{pH}$  and  $\Delta\psi$  in chromaffin granules defines, according to Mitchell's chemiosmotic theory, the existence of a  $\Delta\mu_{\text{H}^+}$ :

$$\Delta\mu_{\text{H}^+} = \Delta\psi - 2.3 (RT/F) \Delta\text{pH}$$

where R is the gas constant ( $8.3 \text{ J.K}^{-1}.\text{mol}^{-1}$ ), T is the temperature in Kelvins and F is the Faraday constant ( $96,494 \text{ J.V}^{-1}.\text{mol}^{-1}$ ). This equation denotes the interconvertibility of the concentration and electrical components. If, as denoted above, the internal pH of the chromaffin granule is around 5.5 regardless of the external pH and, in the presence of MgATP, the transmembrane potential reaches -80 mV, it is readily calculated that  $\Delta\mu_{\text{H}^+}$  across the chromaffin granule membrane at physiological pH ( $\approx 7.4$ ) is close to 200 mV. Apps *et al* (1980*a*) studied the contribution of both factors to the catecholamine uptake showing that transport could be driven by an imposed  $\Delta\text{pH}$  in the absence of a  $\Delta\psi$ , but the rate of uptake was increased by the superimposition of a membrane potential.

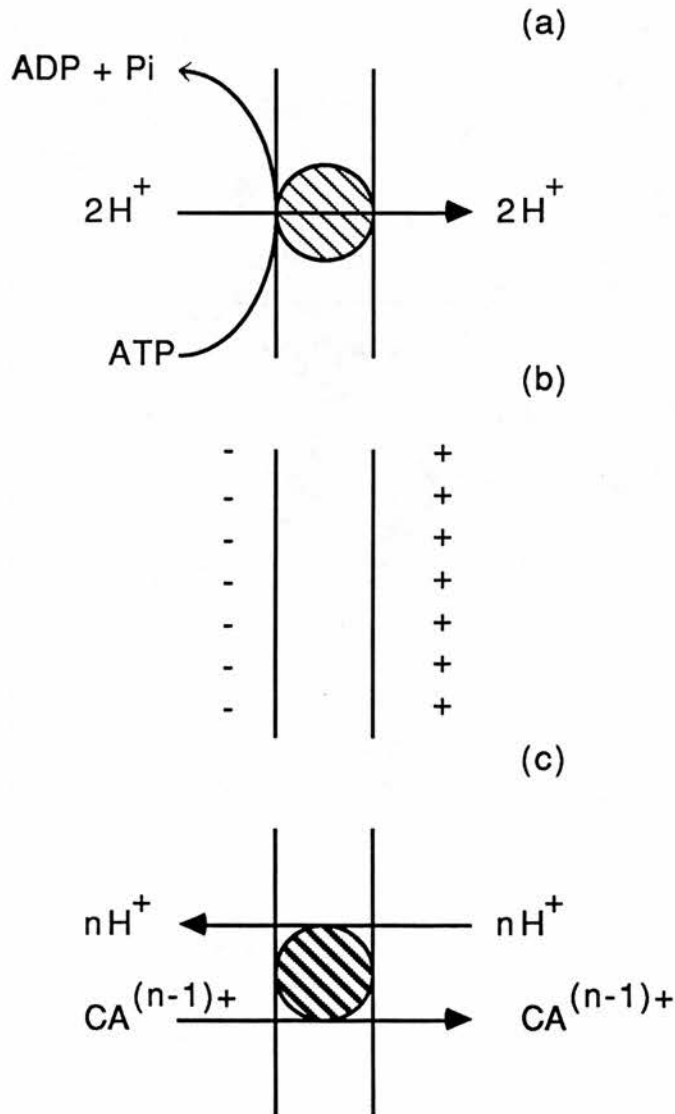
The results of Bashford *et al.* (1975*a,b* and 1976) further supported the idea that the catecholamine uptake occurred via a reserpine-sensitive carrier driven by the proton gradient. The stoichiometry of this process was found to be  $2\text{H}^+$  per protonated amine (Phillips and Apps, 1980; Johnson *et al.*, 1982). According to this, the equilibrium amine concentration gradient depends on the  $\text{H}^+$  concentration gradient and on the membrane potential as follows (see Figure 1.2):

$$\log([\text{CAH}^+]_{\text{in}}/[\text{CAH}^+]_{\text{out}}) = 2\Delta\text{pH} - F\Delta\psi/2.3RT$$

Given a pH gradient of 1.9 units and a membrane potential of -80 mV this equation predicts a catecholamine activity ratio of approximately 140,000:1 which is consistent with the values of concentration found inside and outside the granule.

Cytoplasm

Matrix



**Figure 1.2. Mechanism of catecholamine uptake in chromaffin granules.** (a) The ATPase couples the ATP hydrolysis to  $H^+$ -translocation, thus creating an transmembrane electrochemical gradient of protons (b); (c) the amine carrier exchanges protons for catecholamine molecules (for stoichiometry of this process see below).

Based on the total concentration of soluble components within the chromaffin granules, the osmolality of the matrix should be much higher than the cytosolic osmolality. Since chromaffin granules behave as perfect osmometers (Morris *et al.*, 1977), it has been proposed that ATP, an anion, interacts with protonated catecholamines, probably in the presence of other ions and proteins, to form an intragranular storage complex. The existence of these complexes has been studied using a variety of approaches and techniques and in the final analysis, physicochemical measurements indicate that some interaction of catecholamines at pH 5.6 produces non-ideal behaviour and that the osmotic pressure is one third of that predicted from the intragranular concentrations (Johnson, 1988). These results suggest that the granules use ATP in order to decrease the osmotic pressure inside enabling the accumulation of such a huge amount of catecholamines.

### **1.2.3. Properties of amine uptake.**

The introduction of the resealed chromaffin granule "ghosts" was an important breakthrough in the studies on the catecholamine uptake by chromaffin granules (Phillips, 1974a). Use of "ghosts", formed by hypotonic lysis of the granules, overcomes the complications arising from the presence of existing gradients, large concentrations of endogenous catecholamines and ATP and intragranular binding sites. On the other hand, chromaffin granule ghosts also have some disadvantages compared to intact granules; for example, ATP uptake appears to have different properties in "ghosts" and intact granules (Grueninger *et al.*, 1983). Catecholamine uptake by ghosts exhibits the following characteristics: (1) ATP dependence, (2) structural and steric specificity, (3) saturation kinetics, (4) specific inhibition of transport by various compounds such as reserpine.

#### **1.2.3.1. Specificity.**

In early studies dopamine, noradrenaline, adrenaline and 5-hydroxytryptamine were all shown to be substrates of the catecholamine translocator. It is generally agreed that: (1) hydroxylation of the catechol ring is required for accumulation; (2) carboxylated precursors do not accumulate (Johnson, 1988); (3) uptake is stereospecific, the  $S_{0.5}$  value for (R)-noradrenaline being four times smaller than that of (S)-noradrenaline (Phillips, 1974b).

#### **1.2.3.2. Kinetic studies.**

Kinetic analysis reveals (1) that catecholamine accumulation displays Michaelis-Menten kinetics, (2) that, of catecholamines, dopamine ( $K_m = 16 \mu M$ ) is the preferred substrate and (3) that adrenaline and noradrenaline ( $K_m = 35 \mu M$ ) are transported at the same rate (Carty *et*



*al.*, 1985). The external pH has a marked effect on the kinetic parameters of catecholamine uptake. The  $K_m$  for uptake decreases with the alkalization of the outside pH over the range 6-7.8. This observation supports the hypothesis that the unprotonated species of the catecholamine is transported. Temperature dependence of [ $^{14}\text{C}$ ]adrenaline uptake has been measured with a  $Q_{10}$  of 2.8 from 20 to 30 °C (Kirshner, 1962) and a  $Q_{30}$  of 6.9 from 0 to 30 in isolated granules (Slotkin, 1973).

### 1.2.3.3. Inhibition.

Catecholamine uptake can be inhibited by competitive inhibitors of the transporter, which can be either transported themselves or not, or by perturbation of the electrochemical gradient.

Reserpine became the first established inhibitor of the catecholamine transporter (Kirshner, 1962; Bashford *et al.*, 1976). It is a very hydrophobic compound, a property that seems to be important for its activity. The inhibitory potency of reserpine derivatives diminishes with increased polarity, indicating that the reserpine binding site is hydrophobic (Parti *et al.*, 1987). This hydrophobicity causes some problems, thus, at high concentrations reserpine shows a detergent-like action inhibiting some membrane functions (Zallakian *et al.*, 1982). Moreover, nonspecific binding to the vesicle membrane complicates binding studies. However, it has been shown that there is specific binding of reserpine to chromaffin granules (Weaver and Deupree, 1982) and that this binding is enhanced by ATP and inhibited by the uncoupler FCCP. Two explanations for this effect have been presented (Winkler *et al.*, 1986): one is that reserpine binding could occur only in the inner face of the vesicle and the drug would have to be uptaken first, a process that would be accelerated by ATP. The other explanation is that the electrochemical gradient of protons created by ATP could affect the membrane so that the reserpine binding sites were unmasked.

It has also been reported that there are two distinct populations of reserpine-binding sites (Scherman and Henry, 1983a and 1984), the first (R1) having a binding site density of 7 pmol/mg of protein and a  $K_D = 0.5\text{-}0.7$  nM. The second group of sites (R2) had a lower affinity for reserpine ( $K_D = 20\text{-}25$  nM) and a binding site density of 60 pmol/mg protein.

Reserpine acid, which is an impermeant derivative of reserpine, has been shown to inhibit transport when added externally but not when trapped inside chromaffin "ghosts", which would suggest that reserpine binds mainly to the binding sites facing the cytosol. Moreover, derivatives of tetrabenazine, which is a serotonin analogue, can also inhibit the transporter but, whereas they can displace reserpine from the R2 sites, they do not bind to the R1 sites (Scherman and Henry, 1984). The amine carrier can also be covalently modified and inactivated by the carboxyl-specific reagent DCCD (Gasnier *et al.*, 1985) and diethylpyrocarbonate, which is a histidine modifier (Isambert and Henry, 1985).

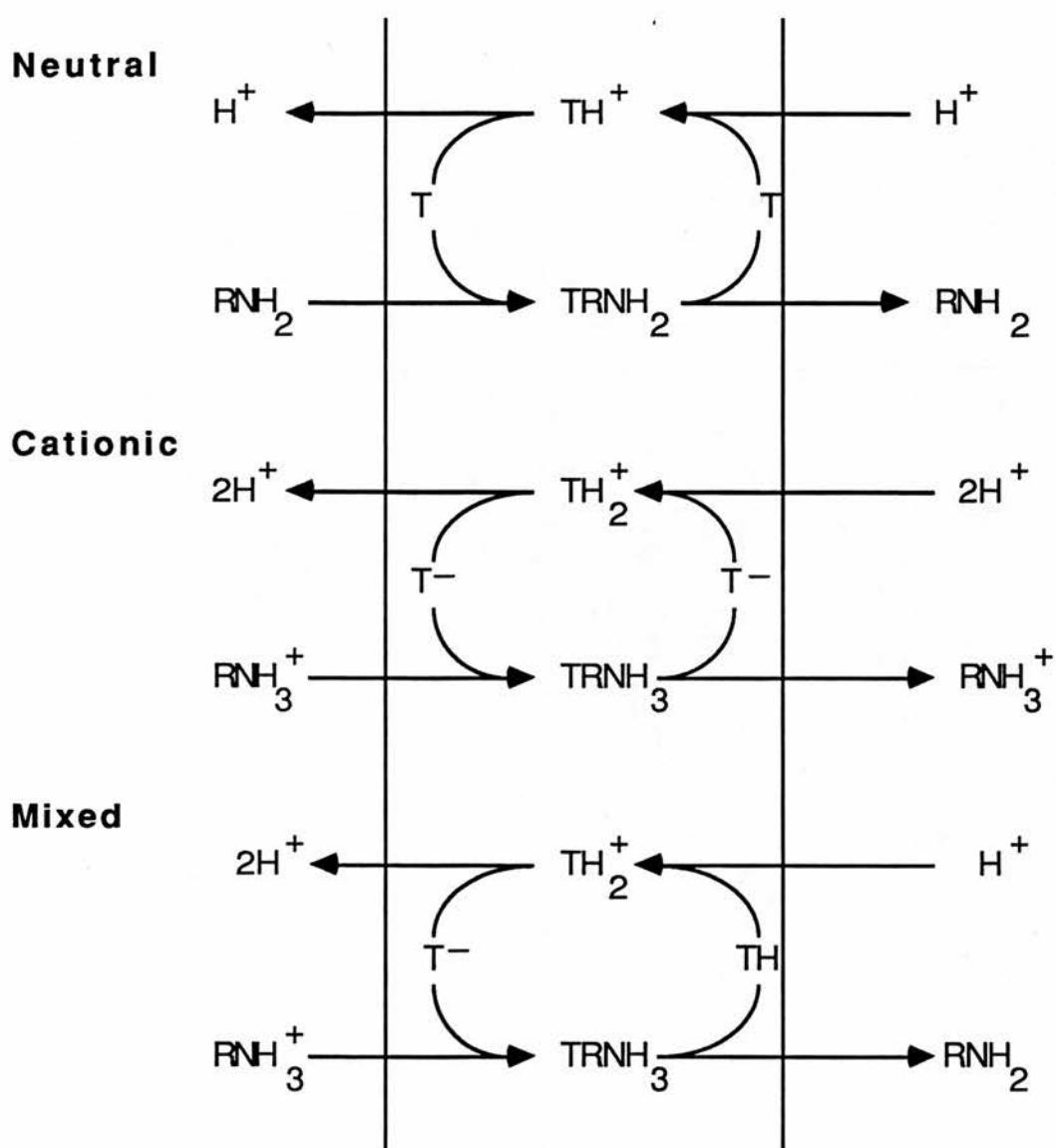
The  $K_D$  values for noradrenaline are 20  $\mu\text{M}$  for the R1 (facing the cytosol) and 2 mM for the R2 (facing the inner membrane). This is perfectly consistent with the  $K_m$  for noradrenaline uptake (20-40  $\mu\text{M}$ ).

#### 1.2.4. Structure of the amine transporter.

The most promising approach to the elucidation of the structure of the amine carrier has been specific photoaffinity labelling. Two probes have been utilized: one is a photoreactive analogue of tryptamine (Gabizon *et al.*, 1982; Gabizon and Schuldiner, 1985) which labels a 45 kDa polypeptide. The binding of this probe is partially inhibited by reserpine and biogenic amines (Gabizon *et al.*, 1982). The other probe used is based on tetrabenazine and it labels a polypeptide of about 75 kDa. The labelling is inhibited by reserpine, nordrenaline and tetrabenazine. Although it has been suggested that R1 sites are located in a 45 kDa protein and R2 in a 75 kDa protein, and that both peptides are subunits of a translocator complex (Henry *et al.*, 1986), this seems implausible because the 45 kDa protein is present in much larger amounts than the 75 kDa (Winkler *et al.*, 1986).

#### 1.2.5. Mechanism of amine transport.

The main point of controversy about the mechanism of amine transport is which form of the amine actually binds to the translocator. The observed  $\text{H}^+$ /amine stoichiometry may be achieved by exchange of one proton for an unprotonated amine or  $2\text{H}^+$  for a protonated amine. The latter mechanism is supported by the fact that the protonated amine is the predominant form at physiological pH (Njus *et al.*, 1986a), however, there seems to be plenty of experimental support for the former, thus,  $K_m$  and  $K_D$  for noradrenaline decrease when the external pH is increased (Scherman and Henry, 1981), a derivative of adrenaline in which the nitrogen carries a fixed positive charge is not transported (Ramu *et al.*, 1983) and dihydrotetrabenazine binds to the translocator in its unprotonated form (Scherman and Henry, 1983b). In any case, there are also arguments against these data: first, the external and internal pH can affect the conformational state of the translocator; a quaternary ammonium complex presents problems of steric hindrance and it is likely that the translocator could bind the amine through a shared hydrogen atom, an interaction that is not possible with a quaternary amine. The fact that tetrahydrobenazine binds to the translocator in its neutral form does not give any support to the idea that the unprotonated species are transported because this compound binds to the internal binding sites (R2) of the carrier (Njus *et al.*, 1986a). This latter observation suggests an alternative mechanism: as we have already seen before, this compound interacts with the internal binding sites of the carrier (R2), thus, it is possible that the translocator could bind the protonated form of the amine on the outside and the neutral form on the inside.



**Figure 1.3. Possible mechanisms of amine transport.** The observed  $\text{H}^+$ /amine stoichiometry may be achieved by the three mechanisms exposed above. See text for further details. (After Njus *et al.*, 1986a).



The latter mechanism would facilitate uptake of easily available protonated amine and would allow only a very small efflux of neutral amines, which are scarce within the granule. This is also consistent with the fact that biogenic amines do not leak out of the acidic interior of chromaffin vesicles at a detectable rate and that the rate of efflux increases as the internal pH is raised (Maron *et al.*, 1983). (see figure 1.3).

#### 1.2.6. Other transport systems in chromaffin granules.

##### 1.2.6.1. ATP.

Nucleotides, mainly ATP, are important components of chromaffin granules in which their total concentration approaches 180 mM, which means a concentration gradient of about 50:1 of nucleotides across the membrane. Some form of active transport has been suggested to explain the existence of this gradient. The main properties of the nucleotide transport in granules are: it is a membrane-potential sensitive, saturable process (Aberer *et al.*, 1978; Kostron *et al.*, 1977; Weber *et al.*, 1983; Weber and Winkler, 1981); the  $K_m$  is in the millimolar range (0.9-1.4 mM); the specificity is broad and includes other negatively charged substrates such as phosphate, sulphate and phosphoenolpyruvate (Weber *et al.*, 1983); it is pH independent.

Great controversy about this issue rises from the fact that the nucleotide transport in "ghosts" shows very different features and seems to be a diffusion effect, non-saturable and independent of the  $H^+$ -ATPase (Grueninger *et al.*, 1983).

Winkler and his colleagues (Weber and Winkler, 1981; Weber *et al.*, 1983) have proposed the existence of an atractyloside-sensitive ATP transport system that would mediate the uptake that ultimately would be driven by the  $H^+$ -translocating ATPase. The existence of a nucleotide carrier is supported by the observation of an atractyloside-sensitive transport of ATP into *Torpedo* cholinergic vesicles, the value of the  $K_m$  being similar to the  $K_m$  found in chromaffin granules. Although the carrier has been stated to be, like its mitochondrial counterpart, a 34 kDa protein that binds atractyloside (Stadler and Fenwick, 1983) and reacts with the photoaffinity label azido-ATP (Luqmani, 1981; Lee and Witzemann, 1983), these results have not been confirmed. Taugner and Wunderlich (1979) reported the existence of a phosphoryl transferase activity in the chromaffin granule membrane that could catalyse the transfer of a phosphoryl group from external ATP to internal ADP, but this activity cannot explain the net uptake of nucleotides.

##### 1.2.6.2. Anion exchange.

Chromaffin granule membranes have a small but measurable permeability to  $Cl^-$  (see above). It has been reported that chromaffin granules can release their contents in the presence

of ATP and 150 mM KCl (Oka *et al.*, 1965). Casey *et al.* (1976) showed that this is due to lysis, provoked by the influx of chloride in response to the membrane potential created by the proton pump; it can be prevented by adding a protonophore, increasing the osmolarity of the medium or replacing chloride by an impermeant anion.

This diffusion and eventual accumulation of chloride within the granule may represent a serious problem *in vivo*, therefore, some workers have sought a mechanism by which the granule can prevent this undesired side-effect of the transmembrane potential.

It has been suggested (Njus *et al.*, 1986a) that chloride could be expelled from the chromaffin granules by chloride/bicarbonate exchange. Due to the internal acidic pH of the chromaffin granule, the concentration of bicarbonate inside the granule is about 30 fold less than in the cytosol, so the  $\Delta$ pH would drive the uptake of bicarbonate in exchange for internal chloride. This mechanism is present in red blood cell membranes and is sensitive to stilbene disulfonates (SITS). The chloride fluxes across the chromaffin granule is also SITS sensitive (Pazoles and Pollard, 1978) but SITS has also been shown to affect the  $H^+$ -ATPase (Pazoles *et al.*, 1981; Cidon and Nelson, 1982). In any case, there is still a lack of experimental evidence about the mechanism of chloride elimination in chromaffin granules.

#### 1.2.6.3. Cation exchange.

Chromaffin granules contain high concentrations of divalent cations although this does not imply that activities are also high; thus the overall intragranular  $Ca^{2+}$  concentration within the chromaffin granule has been calculated to be 17 mM, whereas the activity seems to be the equivalent of a free concentration between 4-24  $\mu$ M (Bulenda and Gratzl, 1986). It has been shown that most of the matrix calcium is bound to ATP, glycosaminoglycans and negatively charged proteins (Winkler and Westhead, 1980; Reiffen and Gratzl, 1986).

Experiments with "ghosts" have shown that a  $Ca^{2+}/Ca^{2+}$  exchange activity is present in chromaffin granules (Phillips, 1981). This exchange was inhibited by  $Na^+$ , ruthenium red (non-competitively) and  $Mg^{2+}$  (competitively). ATP was slightly inhibitory.

$Ca^{2+}/Na^+$  exchange activity, due to the same carrier, was also demonstrated and found to be unaffected by the transmembrane potential generated by the proton pump. This means that the exchange is electroneutral and suggests a stoichiometry of 2  $Na^+/Ca^{2+}$ . Krieger-Brauer and Gratzl (1982 and 1983) obtained similar results. The significance of this activity is not clear although various functions have been proposed (Njus *et al.*, 1986a): it can reduce intravesicular osmotic activity by replacing two monovalent cations with one divalent cation, which might then interact with other matrix components, and the granule might remove calcium from the cytoplasm, this contributing to calcium homeostasis in the cell (Serck-Hanssen and Christiansen, 1973).

Burger and his colleagues have suggested that in intact granules the calcium uptake is ATP-dependent and driven by the transmembrane potential (Häusler *et al.*, 1981; Niedermaier and

Burger, 1981; Burger *et al.*, 1984). If this is the case, three different mechanisms can be proposed (Njus *et al.*, 1986a): first, the  $\text{Na}^+/\text{Ca}^{2+}$  exchange has a stoichiometry of 3:1 or more; second  $\text{Na}^+/\text{Ca}^{2+}$  exchange is coupled with the proton pump via a  $\text{Na}^+/\text{H}^+$  exchange activity; third, chromaffin granule membranes could have a  $\text{Ca}^{2+}$ -translocating ATPase.

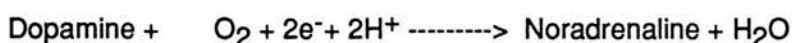
Flatmark *et al.* (1985) provided evidence against the latter alternative by showing that  $\text{Ca}^{2+}$ -ATPase activity was entirely attributable to the proton pump. On the other hand, although Yoon and Sharp (1985) have suggested that the proton gradient is not the direct driving force for  $\text{Ca}^{2+}$  uptake, they could not exclude indirect coupling. Very recently, an amiloride-sensitive  $\text{H}^+/\text{Na}^+$  antiporter activity has been shown to occur in chromaffin granule "ghosts" (Haigh and Phillips, 1989) which gives considerable support to the second alternative.

Another common divalent cation,  $\text{Mg}^{2+}$ , is present in the chromaffin granule matrix at a concentration of 5 mM (Phillips *et al.*, 1977) although its free concentration is not known accurately.  $\text{Mg}^{2+}$  has been reported to occur only in the absence of a proton electrochemical gradient (Feidler and Daniels, 1984), but the role of this ion within the matrix is still unclear.

#### 1.2.7. Electron transfer in the chromaffin granule membrane.

Cytochrome  $\text{b}_{561}$  comprises about 20% of the total protein in the chromaffin granule membrane. Its existence was first reported by Flatmark *et al.* in 1971.

The major reaction known to require electron transfer within the granule is the reduction of dopamine to noradrenaline, which is catalysed by the enzyme dopamine- $\beta$ -monooxygenase (dopamine- $\beta$ -hydroxylase or DBH):



Therefore, from the beginning a link between cytochrome  $\text{b}_{561}$  and DBH was supposed to exist. However, the precise mechanism by which cytochrome  $\text{b}_{561}$  functioned has remained unknown until recently.

A first step towards the elucidation of this role was made by Flatmark and colleagues (Terland and Flatmark, 1975; Ingebretsen *et al.*, 1980) who proposed ascorbate as the most likely electron source for dopamine reduction. This hypothesis was supported by the fact that ascorbate is present within the granule and it is also the most effective electron donor to dopamine- $\beta$ -hydroxylase *in vitro*. On the other hand, Tirrell and Westhead (1979) showed that chromaffin granules do not import ascorbate.

In 1981, Njus *et al.* proposed a model for electron transport across the chromaffin granule membrane based on three basic principles: first, internal ascorbate is regenerated by electron transfer from external ascorbate; second, the  $\text{H}^+$ -ATPase drives this transfer by creating a

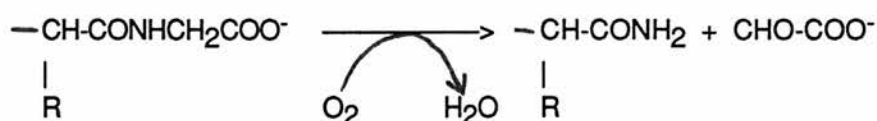
transmembrane electrochemical gradient; third, cytochrome  $b_{561}$  mediates this electron transfer. To this initial theory, some refinements have been added: Njus and colleagues (Njus *et al.*, 1983; 1986a,b) and Wakefield, Cass and Radda (1982; 1986a,b) independently showed that cytochrome  $b_{561}$  mediated a one-electron transfer from the free radical semidehydroascorbate, instead of the fully oxidized ascorbate (dehydroascorbate), to fully reduced ascorbate. This was consistent with the fact that semidehydroascorbate was formed during the reduction of DBH *in vitro* (Diliberto and Allen, 1981; Skotland and Ljones, 1980).

Another refinement that has been suggested is that semidehydroascorbate, formed in the cytoplasm when ascorbate reduces cytochrome  $b_{561}$ , is itself reduced back to ascorbate by the mitochondrial NADH:semidehydroascorbate reductase (Diliberto *et al.*, 1982; 1987; Wakefield *et al.*, 1982; 1986a; 1986b). This proposed model can be explained and justified on thermodynamic grounds (Njus *et al.*, 1986a).

Further evidence in support of this model is that increasing the amount of cytosolic ascorbate in chromaffin cells enhances DBH activity (Levine, 1986) and that oxidation of internal ascorbate by DBH on addition of tyramine can be prevented by adding ascorbate or glycoascorbate to the medium without apparent entry of either compound into the vesicles (Beers *et al.*, 1986; Menniti *et al.*, 1987). Moreover, NADH, glutathione and homocysteine, compounds that cannot reduce cytochrome  $b_{561}$ , do not stimulate noradrenaline synthesis. However, it has also been shown that external ferricyanide, which should not reduce cytochrome  $b_{561}$  (see below), supports DBH activity (Grouselle and Phillips, 1982).

Cytochrome  $b_{561}$  can be oxidized by external ferricyanide and reduced by internal ascorbate (Harnadek *et al.*, 1985; Kelley and Njus, 1986) proving it to be capable of transmembrane electron transfer.

Another role for cytochrome  $b_{561}$  is the supply of electrons to peptidyl glycine  $\alpha$ -amidating monooxygenase, which generates the characteristic  $\alpha$ -amidated C-termini that occur in certain neuropeptides (Kent and Fleming, 1987):



One of the main handicaps for further investigation of the precise mechanism of electron transfer by cytochrome  $b_{561}$  is that no inhibitor for this activity has been found yet.

### 1.2.8. Structure of cytochrome b<sub>561</sub>.

Cytochrome b<sub>561</sub> was identified by Flatmark *et al.* (1971) although Winkler, in the same year, identified a protein that he called chromomembrin B which ultimately proved to be the same protein (Apps *et al.*, 1980b). It is a high potential b-type cytochrome and has an apparent midpoint reduction potential of 140 mV (Flatmark and Terland, 1971). This midpoint potential has also been confirmed for both the membrane-bound and purified cytochrome and shown to be pH independent in the range 5.5-7.5 (Apps *et al.*, 1984).

Chromaffin granule membrane cytochrome b<sub>561</sub> has recently been cloned and sequenced (Perin *et al.*, 1988) and shown to consist of 273 aminoacids with a high average hydrophobicity. The model based on this sequence suggests that cytochrome b<sub>561</sub> spans the membrane six times with the majority of the hydrophilic sequences and the N- and C-termini being cytoplasmic. Using peptide-specific antibodies, Kent and Fleming (1990) confirmed that the C-terminus of the cytochrome is cytoplasmic, but the N-terminus was not located. Clusters of positively charged aminoacids have been observed on the hydrophilic loops of the protein on both sides of the membrane, which may serve to facilitate the interaction of the cytochrome with the negatively charged ascorbate and semidehydroascorbate. As in all b-type cytochromes the h  m group is noncovalently attached. Separate analysis of protein and h  m suggested a h  m to protein ratio of 1:1 (Apps *et al.*, 1984).



### 1.3. CHROMAFFIN GRANULE $H^+$ -ATPase.

The discovery of the chromaffin granule  $H^+$ -ATPase and its role in the creation of an electrochemical gradient of protons across the granule membrane was mentioned earlier. The electrochemical gradient represents an energy store that the granule can use for different purposes: probably the most important one, considering the physiological role of the chromaffin granule, is the uptake of catecholamines against a huge concentration gradient, however, the proton gradient is also involved in  $Ca^{2+}$  movement within the chromaffin cell, electron transfer across the chromaffin granule membrane and possibly in nucleotide uptake; and it is very likely that even more uses remain to be discovered. However, after elucidating the role of the  $H^+$ -ATPase the bulk of ATPase research has been concentrated on the biochemical and structural properties of this protein.

Initial experiments were focused in the solubilization (Trifarò and Warner, 1972), partial purification (Apps and Glover, 1978) and characterization of the biochemical properties of the proton pump in the chromaffin granule membrane by the use of inhibitors. It was shown that a number of inhibitors of mitochondrial ATPase (ATP synthase) had different effects on chromaffin granule ATPase. Thus, DCCD and trialkyltins inhibited proton translocation, ATP hydrolysis and transport; Nbf-Cl and silicotungstate inhibited ATP-dependent activities without affecting the transport driven by an imposed gradient; orthovanadate had a slight inhibitory effect (Apps *et al.*, 1980); oligomycin and efrapoptin did not have any effect on the membrane bound enzyme, whereas on solubilization it became sensitive to efrapoptin (Apps and Glover, 1978). The initial conclusions were that mitochondrial and chromaffin granule ATPases were closely similar as was shown by the fact that both were equally affected by DCCD, Nbf-Cl, trialkyltins and silicotungstate, although they were not identical, since the mitochondrial ATPase inhibitors oligomycin and aurovertin had no effect on the chromaffin granule enzyme. Moreover, it was claimed that the two enzymes showed immunological cross-reactivity (Apps and Schatz, 1979). However, work carried out by Cidon and Nelson (1983) demonstrated that mitochondrial and chromaffin granule ATPases were, in fact, two different enzymes with distinctive features and that the previous results had been biased by the presence of contaminating amount of mitochondrial ATPase in chromaffin granule ATPase preparations.

Nowadays it is accepted that there are three main groups of proton-translocating adenosine triphosphatases, distinguished by their structure, function, mechanism of action and evolution:

- (a) P or  $E_1E_2$ -type, found in fungal plasma membranes and in some specialized cells of higher eukaryotes, such as gastric mucosa.
- (b) F or  $F_1F_0$ -type, found in mitochondria, chloroplasts and bacterial membranes.
- (c) V-type, found in the membranes of acidic intragranular organelles (lysosomes, endosomes, Golgi saccules, secretory vesicles and plant vacuoles). (See Table 1.2)

**Table 1.2. Classification of H<sup>+</sup>-translocating ATPases.**

Type	Source	Approx. M.M.	Subunit types	Inhibitors
F	Bacteria	>450 kDa	8-13	Aurovertin
	Chloroplasts			Efrapeptin
	Mitochondria			Oligomycin
				Venturicidin
P		90 - 140 kDa	1	DCCD
				Nbf-Cl
				Quercetin
V	Fungal plasma membrane	400-700 kDa	3-10	Vanadate
	Gastric mucosa			Nbf-Cl
				Quercetin
	Secretory vesicles			Trialkyltins
	Coated vesicles			NEM
	Lysosomes			Bafilomycin A <sub>1</sub>
	Fungal vacuoles			Diethylstilbestrol
	Plant tonoplasts			Fusidic acid
	Golgi saccules			Suramin
				DCCD
				Nbf-Cl
				Quercetin

### 1.3.1. P-type ATPases.

This class of ATPases is characterized by the formation of a phosphorylated enzyme intermediate during the catalytic cycle. The group includes the  $\text{Na}^+\text{-K}^+\text{-ATPases}$ ,  $\text{Ca}^{2+}\text{-ATPases}$  of both the sarcoplasmic reticulum and plasma membrane, the gastric  $\text{H}^+\text{-K}^+\text{-ATPase}$ , the plasma membrane  $\text{H}^+\text{-ATPase}$  of plants *Neurospora* and yeast and the  $\text{K}^+\text{-ATPase}$  of *E. Coli*. Therefore, amongst this group of enzymes, some carry out coupled transport of two ions whereas others appear to act as unidirectional pumps.

All of these enzymes have a catalytic subunit of molecular mass 90,000 to 140,000, which, in most cases is the only subunit required for ATP-driven cation transport, the only exceptions being the  $\text{Na}^+\text{-K}^+\text{-ATPase}$ , which also contains a 50,000 molecular mass glycoprotein subunit (Forgac and Chin, 1985) and the *E. Coli*  $\text{K}^+\text{-ATPase}$  which requires two additional subunits for ion transport (Laimins *et al.*, 1978). However, it seems likely that all P-type ATPases associate in the plasma membrane in the form of oligomers (Serrano, 1988).

The catalytic subunit is an integral membrane protein that undergoes active site phosphorylation, that is, during each turnover of the enzyme, the terminal phosphate of the ATP is transiently attached to an aspartyl residue in the active site before transfer of the phosphoryl group to water (Bastide *et al.*, 1973). This phosphorylation reaction is stimulated by the presence on the cytoplasmic side of the ion transported from the cytoplasm side to the extracytoplasmic surface. As a consequence of the formation of this intermediate, all these enzymes are inhibited by vanadate which is thought to act as a transition state analogue (Cantley *et al.*, 1977).

Two conformations of the enzyme, called  $\text{E}_1$  and  $\text{E}_2$  (hence the designation of these enzymes as  $\text{E}_1\text{E}_2\text{-type ATPases}$ ), have been identified in animal ATPases, and fungal plasma membrane ATPase also seems to exist in two conformations. The  $\text{E}_1$  conformation is the more sensitive to proteases and thermal inactivation, has its cation-binding site facing the cytoplasm, and is reversibly phosphorylated by  $\text{MgATP}$  but not by  $\text{P}_i$ .  $\text{E}_2$  has the transport site facing the external (extracytoplasmic) side of the membrane and can be reversibly phosphorylated by  $\text{P}_i$ , but not by  $\text{MgATP}$ . The enzyme alternates between the two conformations during its catalytic cycle and this is the basis for the coupling of ATPase activity to cation transport.

Many P-type ATPases have been cloned and sequenced and the results reveal extensive sequence homology even between eukaryotic and prokaryotic enzymes. This suggests that all of them are derived from a common ancestral gene that existed before the divergence between eukaryotes and prokaryotes. All have very similar hydropathy profiles suggesting the presence of 8-10 membrane-spanning  $\alpha$ -helices. Portions of the polypeptides thought to be involved in ATP binding, phosphorylation and the coupling of ion transport to ATP hydrolysis appear to be highly conserved, whereas those regions thought to confer ion selectivity show greater sequence



divergence (Addison, 1986; Hesse *et al.*, 1984; MacLennan *et al.*, 1985).

As far as their physiological function is concerned, although there is rather little experimental evidence, P-type ATPases seem to play a major role in the physiology of the organisms in which they occur like nutrients uptake, ion extrusion and growth control.

For further information see the comprehensive review by Serrano (1988).

### 1.3.2. F-type ATPases.

ATPases of this type occur in mitochondria, chloroplasts and bacteria. They are high molecular mass complexes ( $\approx 500$  kDa) and act by coupling the movement of protons down their electrochemical gradient to the formation of ATP during oxygenic photosynthesis and oxidative phosphorylation. For this reason they are often termed "ATP synthases", which better reflects their physiological role.

F-type ATPases are also called " $F_1F_0$ " because they are composed of two domains:  $F_1$  is a hydrophilic extramembrane domain that contains the ATP hydrolysing activity and  $F_0$  is a hydrophobic membrane-embedded domain which forms the proton channel through the membrane. In most preparations of this type of enzyme these domains can be separated without loss of their respective functions.

Regardless of the source  $F_1$  is composed of five different subunits ( $\alpha, \beta, \gamma, \delta, \epsilon$ ) with a stoichiometry of 3:3:1:1:1 (Schneider and Altendorf, 1987). In *E. Coli* these subunits have molecular masses of 55,000, 50,000, 31,000, 20,000 and 15,000 respectively (Foster and Fillingame, 1982).  $F_1$  is bound to the membrane by interaction with  $F_0$  and in most cases is easily released into the medium by treatment either with EDTA at low ionic strength (Munoz, 1982) or with chloroform (Linnet *et al.*, 1979).

The composition of the  $F_0$  domain varies depending on the source. In bacteria, it is composed of three subunits of molecular masses 19,000-30,000 (a), 13,500-18,000 (b) and 5,500-8,000 (c) with a stoichiometry of 1:2:6-12 (Foster and Fillingame, 1982); in chloroplasts there also seem to be three subunits of 15,500-18,000 (I), 13,000-16,000 (II) and 8,000 (III), although a fourth subunit (IV) of 17,500 appears in some preparations (Pick and Racker, 1979). Subunits IV, I and III in chloroplasts are equivalent to a, b and c respectively in bacteria.

The  $F_0$  domain appears to be more complex in mitochondria. The most striking difference is the existence of a "stalklike" domain containing two subunits of molecular masses 21,000 (oligomycin sensitivity-conferring protein, OSCP) and 9,000 kDa (F6), with OSCP showing homology with subunits b and  $\beta$  in bacteria. In the  $F_0$  domain itself five subunits are found: subunits 9 and 6 show homology with subunits c and a in bacteria, and there are four additional subunits of molecular masses of 5,000-10,000 (subunit 8), 29,000-35,000 (subunit 5),

11,000-15,000 ( $F_b$ ) and 30,000 kDa (uncoupler-binding protein or UBP) (Schneider and Altendorf, 1987).

As far as the function of the different subunits is concerned, subunits  $\alpha$  and  $\beta$  possess nucleotide-binding sites,  $\beta$  being the catalytic subunit and  $\alpha$  the regulatory (Forgac, 1988). The other three subunits of the  $F_1$  domain ( $\gamma$ ,  $\delta$  and  $\epsilon$ ) are thought to be involved in the linking of the  $F_1$  domain to the  $F_0$  domain (Pedersen, 1983). In this latter, subunits c, III and 9 (in bacteria, chloroplasts and mitochondria respectively) most likely form the proton channel. They react covalently with DCCD. Subunits a (IV and 6 in chloroplasts and mitochondria), b (I and OSCP) and subunit II in chloroplasts (no counterpart found in bacteria or mitochondria) apparently serve to stabilize the proton channel although they might be directly involved in  $F_0$  functions, and subunit b (I and OSCP) also seems to be involved in the interaction with the  $F_1$  domain. In mitochondria, OSCP and F6 subunits are required for the binding of  $F_1$  to the membrane sector and for rendering the  $F_1$  complex sensitive to oligomycin and DCCD, subunit 5 is thought to be a contaminant and the functions of subunits 8,  $F_b$  and UBP are unknown (Schneider and Altendorf, 1987).

Virtually all subunits of the F-type ATPases from all sort of sources have been cloned and sequenced and extensive homology found between the corresponding subunits of different pumps. The  $\alpha$  and  $\beta$  subunits also show considerable sequence similarity consistent with a common evolutionary origin (Walker *et al.*, 1984; 1985). In the  $F_0$  domain, subunit a contains five or six membrane-spanning alpha helices and multiple buried polar and charged residues that appear to be important for proton translocation and subunit c is probably composed of two membrane-spanning segments (containing a single buried carboxyl group corresponding to the DCCD-reactive glutamyl residue) connected by a hydrophilic loop which is apparently in contact with the  $F_1$  domain (Walker *et al.*, 1984; 1985). It is noteworthy that an alternative model has been proposed for the subunit c: according to this proposal a third membrane-spanning helix may occur giving rise to a cluster of hydrophilic residues which then can form a "proton wire" (Dunker, 1982).

As far as inhibitors are concerned F-type ATPases show sensitivity to the following agents (see the review by Linnet and Beechey, 1979):

DCCD, dicyclohexylcarbodiimide, reacts covalently with the  $F_0$  domain blocking the proton translocation and preventing ATP hydrolysis. DCCD is a highly hydrophobic compound that can also react with a wide range of groups such as carboxyls, sulphhydryls and tyrosines. As a result DCCD can act as either a condensing agent forming amides or esters, or it can form stable adducts which, in this latter case, implies a covalent binding to the protein. Labelling of  $F_0$  by DCCD is due to the reaction with a conserved carboxyl group in the 8 kDa subunit. However, under some

conditions reaction of DCCD with  $F_1$  subunits is also observed.

Nbf-Cl, 4-chloro-7-nitrobenzofurazan, acts on the  $F_1$  domain, reacting with a tyrosine residue of the  $\beta$ -subunit at neutral pH. At higher pH it can react with thiols in a reaction reversed by sulphydryl reagents; however, intramolecular migration of Nbf-Cl to a lysine can occur at higher pH, rendering the inhibition irreversible.

Efrapeptin is a lipophylic polypeptide that apparently binds to the  $F_1$  domain for it prevents binding of  $P_i$ , competes with both ADP and  $P_i$  and prevents the modification of an arginine residue in  $F_1$  by phenylglyoxal.

Oligomycin inhibits only mitochondrial ATPase and apparently binds to subunit 9. Other inhibitors of this type of ATPases are aurovertin (which binds to the  $\beta$ -subunit) and venturicidin.

### 1.3.3. V-type ATPases.

Acidification of intracellular organelles has been shown to play multiple roles (Forgac, 1988):

(a) In receptor-mediated endocytosis, the acidic pH of endosomes permits ligand-receptor dissociation allowing the cell the recycling of receptor molecules, which can thus be used for multiple rounds of internalization. The low pH of endocytotic compartments has been demonstrated by single-cell fluorescence studies, using fluorescence-labelled ligands. Clathrin-coated vesicles from brain also undergo ATP-dependent acidification, and the V-type ATPase from these vesicles have been the subject of extensive studies (see below), but it seems likely that this ATPase actually originates in neurosecretory vesicles (Moriyama and Futai, 1990; Floor *et al.*, 1990).

(b) In secretory granules, a transmembrane electrochemical gradient of protons drives carrier-mediated uptake of different substrates, the best characterized example being the secretory granules of the adrenal medulla whose bioenergetics are discussed in detail above.

(c) In lysosomes and digestive vacuoles of plant and lower eukaryotes, an acidic internal pH is essential for the activity of hydrolytic enzymes in a variety of endocytotic processes including phagocytosis and pinocytosis. The acidic pH also seems to be involved in the coupled transport of breakdown products from the lysosomes and other digestive vacuoles into the cytoplasmic compartment.

In all these systems the enzymes responsible for creating and maintaining high internal concentrations of protons have been shown to be a novel class of  $H^+$ -translocating ATPases, distinct from P-type and F-type ATPases, called endomembrane, vacuolar or V-type ATPases.

In some situations, V-type ATPases appear in the plasma membrane. In renal tubules, urinary acidification may be partly through the activity of V-type ATPases, which are inserted

into the plasma membrane of tubule cells in response to changes in the acid-base status (Al-Awqati, 1985; Brown *et al.*, 1988). In the midgut of the tobacco hornworm (*Manduca*), a V-type ATPase is involved in secondary transport of K<sup>+</sup> (Schweikl *et al.*, 1989; Wieczorek *et al.*, 1989; 1991). Finally a V-type ATPase has been detected in the ruffled borders of osteoclasts (Vaananen *et al.*, 1990).

#### 1.3.3.1. Inhibitor sensitivities.

One of the important properties of V-type ATPases that helped to demonstrate their distinct nature is their sensitivity to different inhibitors. Thus, unlike P-type ATPases, they are insensitive to vanadate (Forgac *et al.*, 1983; Galloway *et al.*, 1983; Glickman *et al.*, 1983; Gluck *et al.*, 1982; Harikumar and Reeves, 1983; Kakinuma *et al.*, 1981; Percy *et al.*, 1985; Wang and Sze, 1985; Yamashiro *et al.*, 1983). This fact suggests that V-type ATPases do not act by means of a phosphorylated intermediate. Ouabain, another P-type ATPase inhibitor, has been shown to have no effect either in chromaffin granule membranes or "ghosts" (Apps and Reid, 1977; Apps *et al.*, 1980c).

Vacuolar ATPases are also resistant to several inhibitors of F-type ATPases. Oligomycin has no effect on vacuolar ATPases from platelet dense granules (Dean *et al.*, 1984), clathrin coated vesicle (Forgac *et al.*, 1983), Golgi membranes (Glickman *et al.*, 1983), bovine kidney (Gluck and Caldwell, 1987), lysosomes (Harikumar and Reeves, 1983), vacuolar membranes of *Saccharomyces cerevisiae* (Kakinuma *et al.*, 1981) and chromaffin granule membrane (Apps and Glover, 1978; Apps and Reid, 1977; Johnson *et al.*, 1982; Bashford *et al.*, 1976), chromaffin granule "ghosts" and detergent-solubilized membranes (Giraudat *et al.*, 1980). Aurovertin and efrapeptin in chromaffin granules (Apps and Schatz, 1979) or clathrin-coated vesicles (Forgac *et al.*, 1983) also have no effect on ATPase activity.

Amongst the inhibitors of V-type ATPases are Nbf-Cl, N-ethylmaleimide (NEM) and dicyclohexylcarbodiimide (DCCD). These have been shown to inhibit the ATPases from a variety of sources, including clathrin-coated vesicles (Arai *et al.*, 1987a,b; Forgac and Cantley, 1984), *Neurospora crassa* vacuoles (Bowman, 1983), Golgi membranes (Glickman *et al.*, 1983; Zhang and Schneider, 1983), bovine kidney microsomes (Gluck and Caldwell, 1987), turtle bladder (Gluck *et al.*, 1982), lysosomes (Harikumar and Reeves, 1983; Moriyama *et al.*, 1986), vacuolar membranes of *Saccharomyces cerevisiae* (Kakinuma *et al.*, 1981; Uchida *et al.*, 1986) maize tonoplast (Mandala and Taiz, 1986), oat roots tonoplasts (Wang and Sze, 1985) and chromaffin granules.

In chromaffin granules, Nbf-Cl and NEM have been reported to inhibit H<sup>+</sup>-translocation and amine uptake in "ghosts", and the ATPase activity of membranes and purified enzyme (Apps *et al.*, 1980c; Flatmark *et al.*, 1982; 1985; Percy *et al.*, 1985; Percy and Apps, 1986). DCCD inhibition studies have also been carried out on chromaffin granules, "ghosts", membranes and on solubilized and reconstituted enzyme (Bashford *et al.*, 1976; Cidon and Nelson, 1982; Apps *et*



*al.*, 1983 and 1980; Giraudat *et al.*, 1980). The reagent had only a limited effect on solubilized enzyme. DCCD, unlike Nbf-Cl and NEM, also inhibits catecholamine uptake driven by an imposed pH gradient in "ghosts" (Apps *et al.*, 1980c).

Whereas Nbf-Cl appears to inhibit F-type ATPases (Linnet and Beechey, 1979), NEM is quite specific for V-type enzymes. Both inhibitors are alkylating agents that inhibit vacuolar ATPases in an ATP-protectable manner (Arai *et al.*, 1987b; Bowman *et al.*, 1986; Forgac and Cantley, 1984; Mandala and Taiz, 1986; Randall and Sze, 1987; Uchida *et al.*, 1986; Percy and Apps, 1986). Furthermore, Nbf-Cl seems to react with either a tyrosine or a cysteine due to the reversibility of the inhibition by reducing agents such as 2-mercaptoethanol (Forgac and Cantley, 1984; Wang and Sze, 1985).

Inhibition by trialkyltins has also been studied in chromaffin "ghosts" (Apps and Glover, 1978; Apps *et al.*, 1980c) and purified and reconstituted enzyme (Percy *et al.*, 1985; Roisin and Henry, 1982; Giraudat *et al.*, 1980) and these agents have been shown to be potent inhibitors (up to 70% inhibition) with tributyltin as the most potent. However, trialkyltins also inhibit other types of ATPases. Quercetin, a plant flavone, is a non very specific inhibitor that seems to inhibit all types of ATPases (Linnet and Beechey, 1979; Percy *et al.*, 1985).

Recently, several new inhibitors have been found for vacuolar ATPases. Diethylstilbestrol, a non-steroidal synthetic estrogen has been shown to inhibit both ATP hydrolysis and H<sup>+</sup>-translocation in chromaffin granule "ghosts" (Gronberg and Flatmark, 1988), although it also inhibits other types of ion-motive ATPases (Pedersen and Carafoli, 1987; McEnery and Pedersen, 1986). Bafilomycin A<sub>1</sub>, a macrolide antibiotic, has been shown to have a strong and specific inhibitory effect on vacuolar ATPases with very little effect on P-type ATPases and none at all on F-type ATPases (Bowman *et al.*, 1988c; Umata *et al.*, 1990). Although it was speculated that the inhibitor may bind to the catalytic subunit (see below) this conjecture was not based on very strong evidence and the site of inhibition is still unconfirmed. Moriyama and Nelson (1988b) have reported inhibition of vacuolar ATPases by fusidic acid and suramin. Fusidic acid inhibited H<sup>+</sup>-translocation in chromaffin granules, lysosomes, clathrin-coated vesicles and purified, reconstituted chromaffin granule ATPase. Rather less inhibition was observed in plant vacuoles. Suramin inhibited in parallel both H<sup>+</sup>-translocation and ATP hydrolysis in chromaffin granules. F-type ATPases were less sensitive to both inhibitors.

#### 1.3.3.2. Identification of two ATPases in chromaffin granules.

In 1983, Apps *et al.* showed the presence of two different peaks of ATPase activity when solubilized chromaffin granule membranes were eluted through an exclusion column or subjected to centrifugation through glycerol gradients. Later, Percy *et al.* (1985) characterized two activities that they separated by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fractionation after solubilization with detergents and by phase segregation in Triton X-114. One of the ATPases, called ATPase I had an

apparent molecular mass >400 kDa and was resistant to vanadate and efrapeptin and sensitive to DCCD, Nbf-Cl, NEM, tributyltin and quercetin. The other ATPase, called ATPase II, was sensitive to vanadate and quite resistant to the rest of inhibitors. It was concluded that ATPase I was a V-type ATPase involved in vesicle acidification and catecholamine uptake and ATPase II was a P-type ATPase of unknown function, although Zachowski *et al.* (1989) have since suggested that it may be involved in aminophospholipid translocation within the granule membrane.

More recently, the results obtained in chromaffin granules were confirmed by Moriyama and Nelson (1988) and also, Xie *et al.* (1989) found a vanadate sensitive ATPase in the membrane of bovine brain clathrin-coated vesicles. Secretory vesicles from the pituitary were also reported to contain two ATPases whose properties correspond to those in chromaffin granules (Russell, 1984).

#### 1.3.3.3. Subunit composition of V-type ATPases.

Vacuolar ATPases from many different sources have now been purified, but the subunit composition of these enzymes is still controversial (see Table 1.3).

The first purifications were of vacuolar ATPases from plants and lower eukaryotes. They are apparently rather simple enzymes and were originally reported to contain only three subunit types of around 70, 60 and 16 kDa respectively (Bowman *et al.*, 1986; Mandala and Taiz, 1986; Manolson *et al.*, 1985; Randall and Sze, 1986; Uchida *et al.*, 1985). However, in recent work carried out by several groups, preparations of higher purity from higher plants and yeast have been found to contain additional subunits: Parry *et al.* (1983) have claimed that the vacuolar ATPase of higher plants tonoplasts contains seven polypeptides, in yeast vacuolar ATPase eight subunits have been reported (Kane *et al.*, 1989) and in mung bean hypocotyls the enzyme seems to be composed of ten polypeptides (Matsuura-Endo *et al.*, 1990).

Amongst the enzymes from animal sources, ATPase from clathrin-coated vesicles have been reported to contain nine different subunits (Arai *et al.*, 1987a,b; 1988; Xie and Stone, 1986), which form a single macromolecular complex that can be separated from a detergent-solubilized preparation by immunoprecipitation with monoclonal antibodies (Arai *et al.*, 1987a,b). The proton pump from the luminal membrane of kidney epithelial cells reportedly contain ten polypeptides (Gluck and Caldwell, 1987). Lysosomal and Golgi ATPases have also been characterized and shown to be quite similar (Moriyama and Nelson, 1989b,c).

The composition of the chromaffin granule ATPase I is still a matter of controversy. Nowadays it is accepted that at least five subunits are essential for activity. The molecular masses of these polypeptides are 115-120, 72, 57, 40 and 16 kDa (Cidon and Nelson, 1983; 1986; Moriyama and Nelson, 1987a,b; Percy *et al.*, 1985). The biggest subunit (120 kDa) was originally thought to be an aggregate (Percy *et al.*, 1985) but it is now apparent that its frequent absence from polyacrylamide gels is due to its protease sensitivity and to its tendency to aggregate on boiling in SDS.

**Table 1.3. Peptide composition of vacuolar type ATPases.**

Source	Peptide Molecular Masses (kDa)					Ref.
<i>Neurospora</i>		70	62		15	(a)
Yeast	100	69	60	42, 36, 32, 27	17	(b)
Mung bean hypocotyls		68	57	44, 43, 38, 37, 32	16, 13, 12	(c)
Beet tonoplasts	100	67	55	52, 44, 32	16	(d)
Chromaffin granules	115	72	57	40, 33 (?)	16	(e,f,g,h)
Clathrin-coated vesicles	116	70	58	40, 38, 34, 33	19, 17	(i,j,k)
Kidney epithelial cells	115	70	56	45, 42, 38, 33, 31	15, 14, 12	(l)
Lysosomes		72	57	41, 39, 34, 33	18, 15	(m)
Golgi		72	57	41, 34, 33, 30	16	(n)

(a) Bowman *et al.*, 1986.

(b) Kane *et al.*, 1989.

(c) Matssura-Endo *et al.*, 1990.

(d) Parry *et al.*, 1989.

(e) Cidon and Nelson, 1983.

(f); (g) Moriyama and Nelson, 1987*a,b*.

(h) Percy *et al.*, 1985.

(i); (j) Arai *et al.*, 1987; 1988.

(k) Xie and Stone, 1986.

(l) Gluck and Caldwell, 1987.

(m); (n) Moriyama and Nelson, 1989*b,c*.

Another subunit of 33 kDa has been proposed to form part of the complex (Percy *et al.*, 1985); the significance of this was disputed by Nelson's group, but recently they cloned a gene encoding a 32 kDa protein from the ATPase although this protein had an apparent molecular mass of 39 kDa on SDS gels.

Only three subunits (around 70, 60 and 16 kDa) have been found to be common to all preparations of vacuolar ATPases, and immunological evidence suggests that they are highly conserved. Thus, antibodies raised against the SDS-denatured 67 and 57 kDa subunits from beet tonoplasts cross-reacted with subunits of similar molecular masses from chromaffin granules, clathrin-coated vesicles and yeast vacuolar membranes (Manolson *et al.*, 1989). These results suggest common structural features and a common ancestor for vacuolar ATPases of different organelles and different kingdoms.

Immunological cross-reactivity has also been shown to occur between the 72, 57 and 34 kDa subunits from chromaffin granules and the corresponding subunits in lysosomes and Golgi apparatus (Moriyama and Nelson, 1989b,c). Recently, genes encoding the 70 and 60 kDa subunits of ATPases from *Neurospora*, yeast, *Arabidopsis* and carrot have been cloned and sequenced (Bowman *et al.*, 1988a; 1989d; Zimniak *et al.*, 1988; Manolson *et al.*, 1988; Nelson *et al.*, 1989). These two subunits have 60-70% identity in plants and fungi; furthermore, about 25 % similarity was found when the V-type ATPase subunits were compared with their supposed counterparts in F-ATPases (i.e., the 70 kDa subunit with  $\beta$  and the 60 kDa with  $\alpha$ ). Interestingly, the H<sup>+</sup>-ATPases of archaebacteria show closer structural similarity to V-type ATPases than to F-type (Konishi *et al.*, 1990).

#### 1.3.3.4. Subunit function.

##### 1.3.3.4.1. 100-120 kDa subunit.

The presence of this polypeptide is still a matter of controversy. It was originally found only in chromaffin granules (Percy *et al.*, 1985; Moriyama and Nelson, 1986) and clathrin-coated vesicles (Arai *et al.*, 1987a,b; Xie and Stone, 1985). However, this subunit is easy to miss due to its sensitivity to proteases and its tendency to aggregate at high temperatures. Recently, high purity preparations of vacuolar ATPases from yeast and higher plants were reported to contain a subunit of around 100 kDa (Parry *et al.*, 1989; Kane *et al.*, 1989) and Gillespie *et al.* (1991) found a 115 kDa polypeptide in a fraction of renal tubules enriched in vacuolar H<sup>+</sup>-ATPase. This subunit cross-reacted with an antibody raised against the 120 kDa subunit of the chromaffin granule ATPase.

The function of this subunit is unknown although some authors have claimed that it has nucleotide binding sites (Moriyama and Nelson, 1987b). On the other hand, Xie and Stone (1988) have shown that the purified, reconstituted ATPase from clathrin-coated vesicles shows Ca<sup>2+</sup>- and Mg<sup>2+</sup>-dependent ATP hydrolysis and only Mg<sup>2+</sup>-dependent H<sup>+</sup>-translocation. On



removal of the 116 and 17 kDa subunits  $\text{Ca}^{2+}$ -dependent ATP hydrolysis functions as usual but  $\text{Mg}^{2+}$ -dependent ATP hydrolysis or  $\text{H}^{+}$ -translocation are abolished. These results would suggest that the 116 kDa subunit is crucial for the coupling of both activities.

Oligonucleotide probes designed from proteolytic peptide sequences from this subunit in bovine brain clathrin-coated vesicles have been used to isolate partial bovine cDNA clones. Sequences from these were then utilized to isolate rat brain cDNA clones containing the full length coding region. The cDNA sequence predicts a molecular mass of about 96 kDa and the hydropathy profile suggests that it consists of two fundamental domains: a hydrophilic amino-terminal half and a carboxyl-terminal half that contains at least six transmembrane regions (Perin *et al.*, 1991).

#### 1.3.3.4.2. 72 and 57 kDa subunits.

A subunit of 67-72 kDa appears in all the preparations of vacuolar ATPases reported so far. It contains a nucleotide binding site uniquely labelled by NEM in an ATP-protectable fashion in chromaffin granules (Percy and Apps, 1986; Moriyama and Nelson, 1987b), clathrin-coated vesicles (Arai *et al.*, 1987b) and *Neurospora* vacuoles (Bowman *et al.*, 1986). Also ATP-protectable labelling of this subunit by Nbf-Cl has been reported in clathrin-coated vesicle (Arai *et al.*, 1987), *Neurospora* (Bowman *et al.*, 1986), plants (Mandala and Taiz, 1986; Randall and Sze, 1986) and yeast (Uchida *et al.*, 1986). It has around 25% similarity with  $\alpha$  and  $\beta$  subunits from F-type ATPases (Bowman *et al.*, 1988d; Zimniak *et al.*, 1988) believed to be regulatory and catalytic subunits respectively. Moreover, the conservation is extremely high in regions believed to be involved in nucleotide binding. On the basis of this evidence the 72 kDa subunits of vacuolar ATPases are considered the ATP-hydrolysing subunits.

The 57 kDa subunit also appears in all vacuolar ATPases preparations reported so far. Interestingly, this subunit is labelled by the photoactivated ATP analogue 3-O-(4-benzoyl)-benzoyl-adenosine-5'-triphosphate (Manolson *et al.*, 1985), moreover, studies carried out by Bowman *et al.* (1988a,d) revealed a 25% sequence similarity with the 72 kDa subunit. These results support the idea of this subunit having a nucleotide binding site, and it is therefore believed to be the regulatory subunit.

Further support for the suggested distribution of functions between these two subunits came from studies by Mandala and Taiz (1986) in which antibodies raised against the 72 kDa subunit inhibited ATP hydrolysis whereas the anti-58 kDa subunit antibody did not. However, in a review by Stone *et al.* (1990) a more complicated model is proposed. According to this model the hydrolytic centre would be located within the interface of the 72 and 58 kDa subunits, the 40 and 33 kDa subunits (see below) activating the reaction site.

#### 1.3.3.4.3. 30-40 kDa subunits.

Depending on the source of the ATPase, different amounts of subunits around 30-40 kDa are found and there is some controversy about their occurrence and function. Arai *et al.* (1988) found four subunits of molecular masses 40, 38, 34 and 33 kDa respectively when they immunoprecipitated the clathrin-coated vesicle ATPase, and structural studies (see below) suggested that they were sandwiched between the hydrophobic and the hydrophilic domains of the enzyme.

Wang *et al.* (1988) have reported the cloning and sequencing of a 32 kDa protein from chromaffin granule ATPase that shows an apparent molecular mass of 39 kDa on SDS gels. By cross-reaction of antibodies raised against the original polypeptide and a synthetic one they showed that this subunit appears in all vacuolar ATPases from animal sources. On the other hand, Hirsch *et al.* (1988) have cloned and sequenced the gene encoding the 31 kDa subunit from bovine kidney vacuolar ATPase that showed a wide variation in the immunoreactivity of this subunit in different tissues.

However, Nelson *et al.* (1990) have claimed that two polypeptide fragments derived from the 33 kDa subunit from chromaffin granules have the same sequence as the one from bovine kidney and Foury (1990) has reported that a 26.6 kDa subunit from the V-ATPase of *Saccharomyces cerevisiae* exhibits a 34% sequence homology with the E subunit (31 kDa) from bovine kidney microsomes. Furthermore, in the latter work it was also reported that a mutant whose gene encoding this subunit had been inactivated showed no vacuolar ATPase activity. Nelson *et al.* (1990) have cloned and sequenced a protein of about 43 kDa in bovine chromaffin granule ATPase that was shown to be almost identical to its human counterpart. Hydropathy plots reveal that these subunits are quite hydrophilic.

It is generally accepted, although there is a lack of evidence to support it, that this group of subunits are involved in the attachment of the two enzyme domains and coupling of ATP hydrolysis with proton translocation.

#### 1.3.3.4.4. 16 kDa subunit.

The main characteristic of this subunit is its hydrophobicity, which has enabled its purification by organic solvent extraction. It was first identified as the DCCD-reactive component of the vacuolar ATPases in chromaffin granules (Sutton and Apps, 1981) and clathrin-coated vesicles (Arai *et al.*, 1987a; Sun *et al.*, 1987). The labelling by DCCD of a 16 kDa subunit has also been observed in *Neurospora* (Bowman, 1982), plants (Kaetsner *et al.*, 1988; Mandala and Taiz, 1986; Manolson *et al.*, 1985; Randall and Sze, 1986; Rea *et al.*, 1987) and yeast (Uchida *et al.*, 1985). In addition to this, the purified subunit from clathrin-coated vesicles has been reconstituted into liposomes and shown to act as a proton-conducting channel (Sun *et al.*, 1987).

The amino acid sequence of this subunit in the chromaffin granule ATPase has been determined from a cDNA clone, and hydropathy plots revealed four transmembrane segments, one of which contains a glutamate residue that is the likely candidate for the DCCD-reactive site (Mandel *et al.*, 1988). These results support the idea that it forms the proton pore across the membrane, similarly to the 8 kDa DCCD-reactive subunit from F-type ATPases. Interestingly, proton transport has been shown to be completely abolished by reaction of only one subunit with DCCD. This suggests that a functional proton pore requires the cooperative interaction between all the copies of this polypeptide present in the enzymic complex.

Remarkably, a 16 kDa protein isolated from gap-junctions in several tissues (Finbow *et al.*, 1983a; 1983b; Buultjens *et al.*, 1984) has been shown to be identical with this V-ATPase subunit (Dermietzel *et al.*, 1989). It is not yet clear whether it occurs as a contaminant in gap-junction preparations or is a real case of a protein with two different roles (Finbow and Pitts, 1981). A 15 kDa protein isolated from *Torpedo* electric organs, and postulated to be involved in  $\text{Ca}^{2+}$ -dependent release of acetylcholine, also shows sequence homology with this protein (Birman *et al.*, 1990).

Hanada *et al.* (1991) have recently cloned a cDNA for the 16 kDa subunit of a vacuolar ATPase from mouse cerebellum. The deduced polypeptide sequence was shown to have extensive identity with the 16 kDa subunit from chromaffin granule and yeast ATPases, and also with the 15 kDa protein from *Torpedo* (91.6, 72.8 and 89% respectively).

#### 1.3.3.5. Subunit arrangement.

There is a general agreement in suggesting that vacuolar ATPases have a structure similar to that of F-type ATPases, with a hydrophilic domain located outside the membrane and a membrane-embedded hydrophobic domain. The former would consist of 2-3 copies of each of the two subunits of around 70 and 60 kDa, and would constitute the ATP hydrolysing domain, and the latter by several copies of the 16 kDa subunit that would form the proton channel.

Arai *et al.* (1988) carried out topography studies on the clathrin-coated vesicles using membrane-impermeant reagents ( $\text{Na}^{125}\text{I}$ /lactoperoxidase and  $^{125}\text{I}$ -sulphosuccinimidyl-3-(4-hydroxy phenyl)propionate) and the hydrophobic reagent 3-(trifluoromethyl-3-(m-[ $^{125}\text{I}$ ]iodophenyl)diazirine (TID). The results of these experiments indicated that the principal polypeptide labelled from the cytoplasmic surface are those of molecular masses 73 and 58 kDa. TID labelled the 17 kDa subunit most heavily with significant labelling of the 100 and 40 kDa subunits. In subsequent studies carried out by the same group (Adachi *et al.*, 1990a) treatment of the reconstituted  $\text{H}^+$ -ATPase with trypsin resulted in a cleavage of all subunits except the 19 and 17 kDa subunits, consistent with these latter being buried in the bilayer and the former having portions exposed to the cytoplasmic surface. Cross-linking studies (Adachi *et al.*, 1990b) suggested the proximity of the 73 and 58 kDa subunits on one hand and 17, 34, 33 and 40 kDa on the other.

Arai *et al.* (1988) also attempted to determine stoichiometry by quantitative aminoacid analysis. Their results suggested that three copies of both the 73 and 58 kDa subunits are present in the enzymic complex along with six copies of the 17 kDa subunit and one copy of the 100, 40, 38, 34, 33 and 19 Kda subunits. Although careful checks were made on the efficiency of quantitation, some criticisms can be made of this work: first, that the enzyme may already been partially dissociated, and second, that not all subunits may have entered the SDS-polyacrylamide gel used to separate them. If their stoichiometry is correct, the molecular mass of the holoenzyme is very large - over 750 kDa.

In chromaffin granules, incubation of the reconstituted ATPase on ice in the presence of chloride and MgATP resulted in inactivation of the enzyme and release of the 72, 57 and 33 kDa subunits suggesting that these subunits form the cytoplasmic domain. Similar results were obtained with V-type ATPases from several other sources (Moriyama and Nelson, 1989 *a,b,c*; Bowman *et al.*, 1988*b*).

#### **1.3.4. Evolutionary relationships between F-type and V-type ATPases.**

Structural and topographic studies (see above) have shown a high degree of similarity between F-type and V-type ATPases. Moreover, the stoichiometry calculated by Arai *et al.* (1988) further showed a good correlation with that of F-type ATPases (Forgac, 1988). This structural similarity and the idea of a common ancestor for both classes of enzyme have been strongly supported by immunological and genetic studies. Thus, in the previously-mentioned work by Manolson *et al.* (1989), antibodies raised against SDS-denatured 72 and 57 kDa subunits from the *Beta vulgaris* tonoplast ATPase cross-reacted not only with subunits of similar size from vacuolar ATPases but also with the  $\alpha$  subunit of bacterial F-type ATPase. No cross-reaction was observed with F-type ATPases from mitochondria and chloroplasts, suggesting a higher divergence from the common ancestor of these latter enzymes pertaining to higher evolved organisms.

Aminoacid sequence evidence also points in the same direction, thus, Bowman *et al.* (1988*a*; 1988*d*) have cloned and sequenced the genes encoding both the 70 and 60 kDa subunits from the *Neurospora* vacuolar ATPase and showed about 20-25% sequence homology with  $\alpha$  and  $\beta$  subunits from mitochondrial ATPase. Zimniack *et al.* (1988) have also shown a 34.3% sequence homology between the 69 kDa subunit from the carrot vacuolar ATPase and  $\beta$ -subunits of F-type ATPases from different sources. However, Nelson *et al.* (1990) found no homology between the 40 and 33 kDa subunits from chromaffin granules and analogous subunits in the F-ATPase family. They then proposed that the addition of these subunits during evolution defined V-ATPases and F-ATPases as two separate families.

The aminoacid sequence of the 16 kDa subunit of the chromaffin granule ATPase has also been reported (Mandel *et al.*, 1988). In this work, it was shown that 8 kDa subunits of F-type ATPases encoded by organellar or bacterial DNA matched with the first half of the 16 kDa subunit



of the chromaffin granule ATPase, whereas the second half of the latter matched with the 8 kDa subunit of F-type ATPases encoded by chromosomal DNA. It was then proposed that both subunits evolved from a common ancestral gene that existed before the segregation of the various organelles and encoded a 8 kDa protein. The vacuolar ATPase gene probably underwent duplication, the first half evolving in a parallel fashion to bacterial and organellar genes and the second to chromosomal ones. This hypothesis was further supported by cloning and sequencing the gene encoding the same subunit from yeast vacuolar ATPase (Nelson and Nelson, 1989).

In a recent review by Cross and Taiz (1990) a theory of the evolution of ATPases was proposed. According to their hypothesis, both F-type and V-type ATPases are descended from an ancestral complex which first appeared in anaerobic bacteria. The main function of this protein was as a proton pump. It had 12 copies of the proton-conducting subunit (8 kDa) and six of the catalytic subunit (70 kDa). This structure predicts 12 protons pumped per 6 ATP hydrolysed or  $2\text{H}^+/\text{ATP}$ , assuming that in each turnover the number of protons translocated is equal to the number of proton channels and the number of ATP molecules hydrolysed is equal to the number of catalytic subunits.

The evolution of proton-pumping photosystems and electron transport chains allowed the direction of proton flow to reverse, converting the ancestral ATPase into an ATP synthase. When this happened the ratio  $2\text{H}^+/\text{ATP}$  may have been inadequate for maintaining a proper phosphorylation potential. This is illustrated if we consider the thermodynamic relationship between two coupled reactions:

$$\Delta G_{\text{ATP}} = -nF\Delta\mu_{\text{H}^+}$$

When the main function is to synthesize ATP a high value of  $n$  (the  $\text{H}^+/\text{ATP}$  ratio) allows a given  $\Delta\mu_{\text{H}^+}$  to drive the synthesis against a large  $\Delta G_{\text{ATP}}$ ; when the main function is to pump protons, a low value of  $n$  allows a given  $\Delta G_{\text{ATP}}$  to pump protons against a high  $\Delta\mu_{\text{H}^+}$ . It is proposed that when the evolution from the ancestral ATPase to the ATP synthase occurred, the  $\text{H}^+/\text{ATP}$  ratio increased from 2 to a theoretical value of 4 (although experimentally it seems to be 3). This was achieved by duplication of the gene encoding the catalytic subunit (70 kDa) and consequent mutations of one of the resulting genes produced a loss of catalytic activity of three subunits that turned into regulatory subunits (60 kDa).

A second change in the direction of the proton flow occurred when the progenitor of archaebacterial ATP synthase gave rise to eukaryotic vacuolar ATPase. The more appropriate stoichiometry  $2\text{H}^+/\text{ATP}$  was in this case achieved back by duplication of the gene encoding the proton channel followed by fusion and mutation of the new gene that resulted in a loss of one of the two essential carboxyl groups involved in transport. The result was the reduction of 12  $\text{H}^+$ -transporting subunits of around 8 kDa to 6 subunits of around 16 kDa and, therefore, 3 catalytic subunits per 6 proton channels, that is, 2  $\text{H}^+$  transported per ATP hydrolysed.

#### 1.4. AIM OF THE PROJECT.

As we have seen above plenty of work has been carried out on the bioenergetics of chromaffin granules and, although a lot of research still remains to be done, today we have a detailed idea of the physiological role of the chromaffin granule proton pump. However, the structural and biochemical properties of this enzymic complex are not known in detail. The aim of the present research project was to investigate these properties.

As a first step towards this objective, the development of a suitable method for the purification and reconstitution for this enzyme was to be attempted. The idea was to obtain the enzyme quite pure in an environment similar to that found in its native membrane that would allow measurement of its basic function: proton translocation. Once this step was accomplished, the kinetic and regulatory properties, subunit composition, stoichiometry and function of individual subunits within the enzyme could be investigated.

The overall aim was accumulating a body of information about an enzyme pertaining to a group of recently identified  $H^+$ -ATPases. This information would allow us to assess the relationships within this group of enzyme and between vacuolar and F-type ATPases, thus obtaining ideas about the evolution of ATPases in particular and of living matter in general.

**Chapter 2**  
**MATERIALS AND METHODS**



## **2.1. MATERIALS.**

### **2.1.1. Chemicals and Biochemicals.**

Triton X-114 was obtained from Fluka AG, Switzerland; n-octyl- $\beta$ -glucoside was purchased from Sigma and Bio-Gel P6-DG from Bio-Rad. Cholesterol, phosphoenolpyruvate, NADH and nucleotides were supplied by Boehringer, Lewes, East Sussex, U.K, except ATP that was from Sigma. Bovine spinal cord phosphatidylserine and egg phosphatidylethanolamine were obtained from Lipid Products, Nutfield, Redhill, Surrey, U.K. and bovine brain phosphatidylcholine from Avanti Polar Lipids, Inc, Birmingham, USA. Soybean phosphatidylcholine and phosphatidylserine were gifts from Dr. Jörgen Quick, Karlshamns, AB, Stockholm, Sweden; ACMA and Bafilomycin were also generously given by Dr. R. Kraayenhof, Free University, Amsterdam, Holland and Dr. K. Altendorf, University of Osnabrück, Germany. ECL Western blotting detection system was supplied by Amersham International, Bucks, U.K. Folin-Ciocalteu reagent was obtained from BDH. All other chemical were of analytical grade or the highest chemical purity available and were supplied by the Sigma and BDH Chemical Companies, both of Poole, Dorset.

### **2.1.2. Radiochemicals.**

3(-trifluoromethyl)-3-(m-[ $^{125}$ I]iodophenyl)diazirine (TID) (Specific activity > 370 TBq/ml) and Na[ $^{125}$ I] were obtained from Amersham International, Bucks, U.K.

### **2.1.3. Enzymes and proteins.**

Pyruvate kinase (EC.2.7.1.40 from rabbit muscle), lactate dehydrogenase (EC.1.1.1.27 from pig heart) and glycopeptidase F (EC 3.2.2.18) were obtained from Boehringer. Other proteins used were obtained from Sigma: Neuraminidase (EC.3.2.1.18), bovine serum albumin, apoferritin, catalase (EC.1.11.1.6),  $\beta$ -amylase (EC.3.2.1.2), thyroglobulin,  $\beta$ -galactosidase (EC.3.2.1.23), carbonic anhydrase (EC.4.2.1.1), ovalbumin and trypsin inhibitor.

### **2.1.4. Antibodies.**

Peroxidase-linked anti-rabbit IgG antibody was supplied by Sigma. Polyclonal antibodies against the 72 and 57 kDa subunits from *Kalanchoe Daigremontiana* vacuolar ATPase and against the 120 kDa subunit of chromaffin granule ATPase were generous gifts from Mr. M. Warren and Mrs. J. Percy respectively, both of the Department of Biochemistry, University of Edinburgh.

## **2.2. ANALYTICAL METHODS.**

### **2.2.1. Peterson's adaptation of the Lowry protein estimation.**

This method for protein estimation involves the precipitation of proteins with trichloroacetic acid and resuspension of the pellet thus obtained in sodium dodecylsulphate (SDS) and then addition of the Folin-Ciocalteu reagent. This prevents the interference of detergents and reducing agents (Peterson, 1977). BSA was used as a standard and its concentration was checked spectrophotometrically using  $A^{1\%} = 6.6$  at 280 nm.

The assay was carried out as follows: samples and standards were dispensed in microfuge tubes in amounts ranging from 5 to 30  $\mu\text{g}$  of protein and made up to 400  $\mu\text{l}$  in water. 40  $\mu\text{l}$  of 0.15% sodium deoxycholate and, after 5 minutes, 40  $\mu\text{l}$  of 72% TCA, were added. After a further 5 minutes samples were centrifuged at 3,000 for 10 minutes in a Microfuge. Pellets thus obtained were taken up in 400  $\mu\text{l}$  of water and 400  $\mu\text{l}$  of Reagent A (0.2 M NaOH, 2.5% SDS, 2.5% sodium carbonate, 0.05% potassium sodium tartrate and 0.025% copper sulphate pentahydrate) were added. After 10 min incubation at room temperature, 200  $\mu\text{l}$  of a 20% (v/v) solution of Folin-Ciocalteu reagent were added. Absorbances at 750 nm were measured after 30 min at room temperature and a standard curve was derived by plotting absorbances against BSA concentrations. The protein concentration of each sample was calculated from the standard curve.

### **2.2.2. Measurement of ATPase activity.**

Hydrolysis of ATP was assayed spectrophotometrically, by following NADH oxidation in a coupled assay system. Samples were assayed in 1 ml of the following medium: 2 mM ATP, 10 mM  $\text{MgSO}_4$ , 1 mM phosphoenolpyruvate, 0.2 mg/ml NADH, 50 mM KCl, 50 mM Hepes/KOH, pH 7.0, lactate dehydrogenase (3.6 units/ml), pyruvate kinase (3.0 units/ml) and 2  $\mu\text{M}$  FCCP. The oxidation of NADH was followed by monitoring the absorbance change at 340 nm. Assays were performed in plastic cuvettes at 34 or 37°C in a Pye-Unicam SP1800 recording spectrophotometer. The assay was started by addition of the sample (10-40  $\mu\text{l}$ ). ATPase activity was expressed as the rate of conversion of NADH in nmols/min/mg of protein using the extinction coefficient  $\epsilon = 6.22 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ . The assay consists of a series of coupled reactions: ATPase hydrolyses ATP thus producing ADP which is then phosphorylated by phosphoenolpyruvate yielding ATP and pyruvate. This step is catalized by pyruvate kinase. Pyruvate is then reduced by NADH yielding lactate and  $\text{NAD}^+$  in a reaction mediated by lactate dehydrogenase. The decrease of absorbance of NADH at 340 nm is then recorded.

The main advantage of this assay is that ADP is constantly removed. However, it has been argued (Jenkins, 1991) that, since ADP, the product of the initial reaction, is itself the substrate for one of the coupling enzymes (pyruvate kinase), the rate of removal of ADP actually depends on the amount of ADP produced, that is, on the velocity being measured. As a consequence, at low ATPase activities, the steady-state level of ADP might be high enough to exert some inhibitory effect, thus altering the measured rate of ATP hydrolysis. In any case, a huge excess of pyruvate kinase (3 units/ml) with respect to the amount used by Jenkins (0.075 units/ml) is routinely present in the experiments presented in this thesis and, under these conditions, as Jenkins himself reports, the problems produced by the presence of ADP are minimized.

### **2.2.3. ACMA quenching assay.**

This assay was used to measure the proton gradient created by the enzyme reconstituted into sealed proteoliposomes (see Chapter 4). The assay was carried out as follows: about 2  $\mu\text{g}$  of reconstituted protein were added to a medium containing 0.3 M sucrose, 10 mM Hepes-NaOH, pH 7.4 and 0.4  $\mu\text{g/ml}$  valinomycin (total volume 0.5 ml). For standard assays in reconstitution experiments, 0.2  $\mu\text{g/ml}$  of ACMA and 3 mM  $\text{MgSO}_4$  were also present, whereas for kinetic experiments the amount of ACMA was 0.3  $\mu\text{g/ml}$  and  $\text{MgSO}_4$ , 1 mM. Usually a small ATP-independent quench was observed, after which 3 mM ATP (reconstitution experiments) or different amounts of MgATP (kinetic experiments) were added. ACMA quenching was recorded in a Perkin-Elmer 3000 fluorimeter with the cuvette thermostatted at 30°C and  $\text{H}^+$ -translocation was expressed as the initial rate of quench in arbitrary units/min.

The theoretical basis both of the assay itself and of the use of the initial rate of quenching as a measurement of  $\text{H}^+$ -translocation activity will be discussed in detail in Chapter 4 and Appendix 2.

### **2.2.4. Estimation of Triton X-114 concentration.**

The concentration of the detergent Triton X-114 was estimated using the method of Garewal (1973). The procedure is based on the reaction of cobalthiocyanate with the poly(ethylen oxide) groups of the Triton family of detergents. This reaction produces a blue precipitate which is then extracted into dichloromethane. The absorbance of this extract is scanned over the wavelength range 570-700 nm and the absorbance difference between 622 and 687 nm is proportional to the detergent concentration. This assay is calibrated using standard concentrations of detergent and constructing a standard curve over the range 0-400  $\mu\text{M}$  of detergent. A stock solution of 2 mg/ml of Triton X-114 in 50% ethanol was used to give a concentration range as indicated above. The assay volume was made up to 300  $\mu\text{l}$  with 50% ethanol. 0.4 ml of cobalthiocyanate reagent (17.8 g  $\text{NH}_4\text{SCN}$ , 2.8 g  $\text{Co}(\text{NO}_3)_2$  in a total volume

of 100 ml with water) was mixed with each sample and then left for 5 minutes at room temperature for colour development. 1.5 ml. of dichloromethane were then added and each sample was vortexed for 2 minutes. Separation of the two phases was induced by centrifugation at 3,000 r.p.m. in a bench centrifuge ( $g_{av} = 1.5 \times 10^3$ ). The lower solvent layer was scanned in glass cuvettes using a Pye-Unicam SP1800 Split Beam Spectrophotometer with a detergent blank as a reference. Scan speed was 2 nm/sec and recorder speed 1 cm/min. The difference in absorbance between 622 and 688 nm was plotted against detergent concentration to generate a standard curve from which the detergent concentration in the sample was calculated.

#### **2.2.5. Gel electrophoresis.**

Polyacrylamide gel electrophoresis was usually performed using slab gels containing an exponential gradient of acrylamide from 8-15% (where indicated 10% straight gels were utilized). The gel system was taken from the method of Laemmli (1970), with SDS in the separating and stacking gels and mercaptoethanol or dithiothreitol in the sample buffer. Acrylamide and bis-acrylamide used in the gels were of electrophoresis grade.

##### **2.2.5.1. Gel preparation and pouring.**

Glass gel plates (18 x 14 cm) were cleaned with methanol and then clipped together with three PVC spacers (1.5 mm thickness) on either side and along the bottom. One of the gel plates had a 13 x 2 cm slot at the top. The sides and bottom were then sealed with a 1.5% solution of molten agar. "Mini-gels" (8 x 8 cm, 0.75 mm thickness) were also run; in this case the bottom was sealed with 1.5% molten agar but sides were not, being only clipped together.

The separating buffer had the following composition: 0.375 M Tris-HCl pH 8.8, 2 mM EDTA, 0.1% SDS, 0.5% (w/v) polyacrylamide, 0.12% (v/v) TEMED and 8 (A) or 15% (B) acrylamide/bis-acrylamide. 20 ml. of solution A were pumped, using a Gilson multi-channel pump, into a mixing chamber containing 10 ml. of solution B and mixed with a magnetic stirrer. As mixing took place, mixed solution was pumped out to the gel cassette at the same rate (1.5 ml/min). Polymerisation of the gel was initiated by addition of 0.05% (w/v) ammonium persulphate (freshly prepared) to each of the separating solutions before mixing was begun.

The gel was poured to within 3 cm of the top of the gel plates to allow room for the stacking buffer and water-saturated butan-2-ol was layered on top of the gel to give an even surface. After polymerisation of the separating gel, the butanol was rinsed off with distilled water and stacking gel was applied.

The stacking gel had the following composition: 0.125 M Tris-HCl pH 6.5, 2 mM EDTA, 0.1% (w/v) SDS, 4.5% (w/v) acrylamide/0.12% (w/v) bis-acrylamide, 0.5% (w/v) polyacrylamide, 0.125% TEMED. The polymerization was started by addition of 0.156%

ammonium persulphate and the mix was poured on the top of the separating gel. Before the stacking gel set, a gel comb was inserted between the tops of the glass plates to allow formation of wells for sample loading.

#### 2.2.5.2. Sample preparation.

Samples with a high lipid content were precipitated with 10-50 volumes of acetone-ethanol (1:1) to remove lipid. Precipitation took place over 1-2 hours in an ice-salt bath and the protein was collected by centrifugation at 15,000 r.p.m. for 20 min ( $g_{av}=17,600$ , 4°C) and redissolved in sample buffer containing 0.05 M Tris-HCl pH 6.5, 5% (w/v) SDS, 2 mM EDTA, 10% (w/v) glycerol. Protein samples not requiring delipidation were simply redissolved in sample buffer. Mercaptoethanol (0.05% v/v) or 10 mM dithiothreitol, and bromophenol blue (0.005% v/v) were added to all samples at this stage.

#### 2.2.5.3. The gel run.

After the stacking gel polymerized, the gel comb and bottom spacer were removed from the cassette. Paraffin wax was applied to the top half of the slotted glass plate to enable a seal to be formed as the cassette was positioned on the gel tank. The gel tanks were then filled with electrode buffer (0.05 M Tris, 0.38 M glycine, 0.1% (w/v) SDS, 2 mM EDTA) and the sample wells were cleaned of loose acrylamide using a needle and a syringe containing electrode buffer. Any bubbles at the bottom of the gel were also dislodged. Samples were loaded into the sample wells using a Hamilton syringe and gels were run overnight at a constant potential of 60-80 volts.

"Mini-gels" were clipped to a "Bio-Rad Tall Mighty Small" slab gel equipment and comb was removed after the electrode buffer was poured. No loose polyacrylamide or air bubbles had to be dislodged, the bottom of the mini-gel remaining sealed with agar. Samples were loaded as above and running was normally at a constant current of 20 mA for about 2 hours.

#### 2.2.5.4. Fixing and staining of the gel.

When the dye front had reached the bottom of the gel, the tank was disconnected and the gel was removed. The glass plates were separated and the gel placed in fixing solution (10% (v/v) acetic acid, 20% (v/v) methanol) for about 15-30 min, with gentle agitation. The gel was then drained and stained with 0.125% Serva Blue R, 50% (v/v) methanol, 7.5% (v/v) acetic acid for 5-10 min. Destaining was achieved by pouring out the dye and soaking the gel in destaining solution (10% (v/v) methanol, 7% (v/v) acetic acid) in a shaking water bath. A piece of polyurethane foam was included to accelerate the destaining procedure by binding the dye.



#### 2.2.5.5. Silver stain.

The method of Wray *et al.* (1981) was followed. The gel was run and fixed as above. After fixing, the gel was soaked in 50% methanol for three hours with two or three 5 minutes rinses with water in-between. After this, the gel was rinsed again with water and silver solution was added. The silver solution was prepared as follows: 0.4 g of silver nitrate in 2 ml of water were added dropwise on a solution containing 21 ml of 0.36% NaOH and 1.4 ml of 14.8 M ammonium hydroxide and the resulting solution was made up to 100 ml. After 5 min agitating in the silver solution, the gel was again rinsed with water and then developed with about 250 ml of 0.05% (w/v) citric acid, 0.0175% (v/v) formaldehyde. The gel was soaked in the developing solution until the protein bands were visualized, after which development was stopped with gel-fixing solution (see above).

#### 2.2.6. Immune Replica technique for electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets.

The protocol was taken from Towbin *et al.* (1979). Polyacrylamide gels were removed from the gel plates and placed into a "sandwich" consisting of a support, scouring pads, filter paper and a nitrocellulose or Pro-Blot sheet. The transfer cassette was assembled in a tray containing transfer buffer (20 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.02% (w/v) SDS, 20% (v/v) methanol). With the cathode grid of the cassette at the bottom, the "sandwich" was constructed in the following order: scouring pad, filter paper, polyacrylamide gel, nitrocellulose sheet, filter paper and scouring pad. Air bubbles were excluded by submerging the layers in buffer. The cassette was secured with a clip and rubber bands and lifted into the transfer tank, transfer buffer being circulated by means of a pump. The proteins were then transferred for 2 hours with a current of 0.8 A.

#### 2.2.7. Glycerol gradients.

Glycerol gradients were poured in 10% methanol in working buffer (10 mM Hepes-KOH or Tris-HCl, 0.15 M KCl pH 7.4, 1 mM DTT, 0.1 mM EDTA). Different amounts of detergent and lipids were also added as indicated in the respective sections. Gradients were poured from a gradient mixer with the mixing chamber being magnetically stirred, and the solution being pumped into Beckman Ultraclear centrifuge tubes (13.2 or 5 ml) using a peristaltic pump. The concentrations of the mixing solutions were calculated according to the expression:

$$C_b = C_a - (V_t/V_g) \times (C_{\text{bottom}} - C_{\text{top}})$$

where  $C_a$  is the initial concentration in the mixing chamber (the same as the solution in the bottom of the gradient or  $C_{\text{bottom}}$ ),  $V_t$  is the total volume of both mixing chambers,  $V_g$  is the volume of the gradient,  $C_b$  is the concentration in the diluting chamber and  $C_{\text{top}}$  is the concentration at the top of the gradient. Gradients were stored at 4°C for several hours before use.

#### **2.2.8. Estimation of lipid content in protein samples.**

Quantitative estimation of lipid contents in samples from different stages of the chromaffin granule  $H^+$ -ATPase purification (see Chapter 4) was performed at the Cardiovascular Research Unit, Edinburgh University. Cholesterol was estimated using the "Cholesterol C-system" kit from Boehringer. The "Wako Phospholipids B" test was utilized for determination of phospholipids (lecithin, sphingomyelin and lysolecithin). The Boehringer kit was supplied by Boehringer, Lewes, East Sussex, U.K. and the Wako kit by Alpha Laboratories Ltd., Eastleigh, Hampshire, U.K.

### **2.3. GENERAL METHODS.**

#### **2.3.1. Preparation of chromaffin granule membranes.**

Bovine adrenal glands (40-50) were obtained from a local slaughterhouse within one hour of slaughter. The adrenal medullae were dissected from the cortex of each gland and placed in 400 ml. of 0.3 M sucrose/10 mM Hepes-NaOH pH 7.0, at 0°C and all the subsequent operations were performed at 0-4°C. The medullae were minced with a stainless steel mincer and then homogenized with a loose-fitting pestle using a Heidolph homogenizer (1,000 r.p.m., 6 passes). The homogenate was then centrifuged in a Beckman JA 14 rotor for 5 min at 4,000 r.p.m. ( $g_{av} = 1540$ ) and the pellet discarded. The supernatant was centrifuged again at 14,000 r.p.m. for 30 min. in the same rotor ( $g_{av} = 19,000$ ). The supernatant was discarded and the pellet resuspended in buffered sucrose. This material was then centrifuged for 20 min. at 15,000 r.p.m. in a Beckman JA 20 rotor ( $g_{av} = 17,600$ ). The fluffy brown mitochondrial layer was swirled from the pellet with buffered sucrose leaving the crude chromaffin granules behind. This pellet was resuspended and homogenized in buffered sucrose and used as a crude granule preparation.

The crude granule fraction was diluted to a volume of 120 ml with 0.3 M sucrose/Hepes and overlaid on 6 x 50 ml aliquots of 1.6 M sucrose/10 mM Hepes-NaOH pH 7.0, then centrifuged at 45,000 r.p.m. for 60 min in a Beckman Ti45 rotor ( $g_{av} = 158,000$ ). After centrifugation, the mitochondrial layer at the 1.6 M sucrose/supernatant interface was removed



by suction and discarded. The pink chromaffin granule pellet was resuspended in a small volume of 10 mM Hepes-NaOH pH 7.0 by gentle homogenization. This fraction was then diluted further, to a volume of 210 ml to produce granule lysis. Chromaffin granule membranes were separated from the granule lysate by centrifugation at 45,000 r.p.m. for 20 min in the Ti45 rotor. The clear lysate was discarded and the pink membrane pellet was taken up in a small volume of 10 mM Hepes-NaOH pH 7.0. To remove any contaminating mitochondrial membranes that might still be present, the membranes were diluted to 40 ml. and overlaid on 2 x 50 ml aliquots of 1.0 M sucrose/10 mM Hepes-NaOH pH 7.0, then centrifuged at 45,000 r.p.m. in the Ti45 rotor for 30 min. Chromaffin granule membranes were collected from the sucrose/supernatant interface and stored frozen in the presence of 10 mM Hepes-NaOH pH 7.0, 1 mM DTT and 0.1 mM EDTA.

### **2.3.2. Preparation of chromaffin granule "ghosts".**

"Ghosts" were prepared by a modification of the method described by Apps *et al.* (1980). Crude granules (about 20 mg of protein/ml in 0.3 M sucrose-Hepes) were added dropwise to 340 ml of 10 mM Hepes buffer on ice, while stirring. The granules lysed immediately in the hypo-osmotic buffer and, after a few minutes, 60 ml of 2 M sucrose were added to restore the osmolarity to 0.3 M. The resealed "ghosts" were collected by centrifugation at 23,000 r.p.m. in a Beckman Ti45 rotor ( $g_{av} = 42,000$ ) for 30 min; "ghosts" formed a pink outer layer with a darker, central disc of contaminating mitochondrial membranes at the bottom of the tube. The latter were discarded as far as possible and the crude resealed "ghosts" were resuspended by gentle homogenization in buffered 0.3 M sucrose to a final volume of about 20 ml. Centrifugation onto a density gradient step was then used to purify "ghosts" from any remaining mitochondrial contamination. These were prepared in Beckman cellulose nitrate tubes (14 x 89 mm) by underlaying 4.5 ml of 0.4 M sucrose/10 mM Hepes with 2.5 ml of 0.4 M sucrose in D<sub>2</sub>O/10 mM Hepes, using a syringe with a long needle attached. 5 ml of the "ghost" suspension were carefully overlayed onto the upper layer, followed by centrifugation at 40,000 r.p.m. ( $g_{av} = 196,000$ ) for 30 min in a Beckman SW 41 rotor; the pink band of purified "ghosts" (3-6 mg/ml.) was collected from the 0.4 M sucrose/0.4 M sucrose-D<sub>2</sub>O interface and stored in 0.5 ml aliquots at -20°C.

### **2.3.3. Purification of Triton X-114.**

Triton X-114 was precondensed as described by Bordier (1981). 20 g of detergent were added to 10 mM Tris-HCl pH 7.3, 0.15 M NaCl to give a final volume of 1 litre. 16 mg of butylated hydroxytoluene in 100 µl of ethanol were added and the solution was put on ice and left at 4°C until the solution completely cleared. The detergent was then condensed by transferring the solution to a separating funnel and incubating overnight in an oven at 30°C. The detergent

fraction was decanted. This process was repeated twice more and the final detergent fraction was assayed to estimate its concentration (see above).

#### **2.3.4. Fractionation of chromaffin granule membranes by temperature-induced phase separation in Triton X-114.**

The method of Pryde and Phillips (1986) was followed. Chromaffin granule membranes (obtained as described above) in 10 mM Hepes-NaOH pH 7, 1 mM DTT, 0.1 mM EDTA were sedimented by centrifugation in a Beckman TL-100.3 rotor at 100,000 r.p.m. ( $g_{av}$  = 412,000). The membrane pellet were resuspended in 0.15M NaCl/10 mM Tris-HCl, pH 7.6 (Tris/salt buffer, TBS) and Triton X-114 was added at 0°C so that the final concentration of detergent was 20 mg/ml Triton and protein concentration was 4 mg/ml. The mixture was homogenized and kept on ice for 5 min, after which the white pellet (P1) was removed by centrifugation as described above. The pellet was washed by homogenization in the same buffer containing 20 mg/ml of Triton X-114, followed by centrifugation, and finally resuspended in 10 mM Hepes-NaOH pH 7 and kept at -20°C.

The supernatant left after the removal of P1 was layered over 1 ml cushions of 0.25 M sucrose/TBS/0.06% Triton X-114 in conical glass centrifuge tubes. The tubes were incubated at 30°C for 5 minutes to separate the detergent phase followed by centrifugation at 2,500  $g_{av}$  (swing-out bench centrifuge, 4,000 r.p.m.) for 5 minutes at room temperature. The supernatant ("aqueous phase") was removed from above the cushion. The cushion was then carefully removed, the "detergent-rich" phase recovered and resuspended to its original volume in ice-cold TBS.

To remove cross-contaminants from these phases, the above procedure was repeated twice with intermediate incubations on ice for a 5 minutes. Finally, the detergent phase was resuspended to one-fifth original volume in ice-cold TBS for further use.

The residual detergent in the aqueous phase was removed by dialysis against 100 volumes of TBS containing 1% Amberlite XAD-2, 0.2 mM PMSF, 1 mM benzamidine at 4°C for 5 days with two changes of buffer. The dialysed fraction was diluted with three volumes of TBS and centrifuged at 412,000  $g_{av}$  (Beckman TL 100.3 rotor) for 15 minutes at 2°C. The pellet (the "glycoprotein-rich" fraction) was resuspended by homogenization in 10 mM Hepes-NaOH (pH 7.2) with 0.1% Triton X-100. All fractions were stored at -70°C.

## **Chapter 3**

### **PURIFICATION OF THE CHROMAFFIN GRANULE H<sup>+</sup>-ATPase**

### 3.1. INTRODUCTION.

Animal vacuolar ATPases have been isolated from a number of different sources including chromaffin granules (Percy *et al.*, 1985; Cidon and Nelson, 1986; Moriyama and Nelson, 1987*a,b*; Moriyama and Nelson, 1989*a*), clathrin-coated vesicles (Xie and Stone, 1986; Arai *et al.*, 1987), kidney medulla microsomes (Gluck and Caldwell, 1987), Golgi membranes (Young *et al.*, 1988) and lysosomes (Moriyama and Nelson, 1989*b*).

Published purification methods are usually long processes involving chromatography and density gradient centrifugation (Cidon and Nelson, 1986; Moriyama and Nelson, 1987*a,b*; Xie and Stone, 1986; Arai *et al.*, 1987). Furthermore, due to the fact that ATPases are membrane-bound proteins, the presence of detergents is required at every purification step. Normally, once the isolation of the ATPase is accomplished, reconstitution into liposomes is needed for the enzyme to acquire its proper conformation. This usually involves the addition of lipids (except when intrinsic lipids copurify with the enzyme) to the solubilized purified ATPase and removal of detergent by dialysis (Xie and Stone, 1986; Arai *et al.*, 1987) or dilution (Moriyama and Nelson, 1987*a,b*; 1989*a*). However, reconstitution is not always carried out (Cidon and Nelson, 1986). In any case, the isolation of vacuolar ATPases can take several days.

As far as the chromaffin granule H<sup>+</sup>-ATPase is concerned, two groups have reported methods of purification. In 1986, Cidon and Nelson published the isolation of chromaffin granule ATPase with a reported specific activity of 17 units/mg. The procedure involved chromatography on hydroxylapatite and DEAE-cellulose, followed by two glycerol density gradients centrifugation steps. No reconstitution was performed and no H<sup>+</sup>-translocation was assayed. In 1987, Moriyama and Nelson (1987*a,b*) reported a simpler method of purification which involved just one hydroxylapatite column and one glycerol gradient. In this case, reconstitution of the enzyme was achieved by diluting the final solution of enzyme, which contained intrinsic lipids. The specific activity of the final product was 5 units/mg. However, in a following paper (Moriyama and Nelson, 1989*a*) the enzyme purified by the same method had a specific activity of only 3.5 units/mg. Percy *et al.* (1985) also reported purification of the chromaffin granule H<sup>+</sup>-ATPase using two different approaches: first, chromaffin granule membranes solubilized in the non-ionic detergent C<sub>12</sub>E<sub>8</sub> were treated with ammonium sulphate, the enzyme coming out of the solution as a "floating cake". In the second method, the treatment of membranes with the detergent Triton X-114 is utilized. Neither of these two methods yielded a product of high specific activity, however, analysis by SDS-PAGE of the final products suggested a high degree of purity.

The treatment with Triton X-114 allows the separation of membrane proteins according to their hydrophobicity (Bordier, 1981; Pryde and Phillips, 1986). Triton X-114 does not

solubilize all membrane proteins; the most hydrophobic ones are left as a white suspension (P1) that can be centrifuged out of the remaining solution. P1 contains the H<sup>+</sup>-ATPase. The solubilized proteins can be further separated in two phases: at 30 °C Triton X-114 condenses and separates from the aqueous solution, the solubilized proteins being divided between the detergent-rich phase (P2) and the aqueous phase (S2). Removal of residual detergent from this aqueous phase leads to the precipitation of intrinsic glycoproteins, while extrinsic proteins such as the chromogranins remain in solution.

Fractionation with Triton X-114 affords extensive purification of the chromaffin granule H<sup>+</sup>-ATPase in a single step but, as mentioned above, the final product has a low specific activity. In this chapter, an adaptation of the Triton procedure, designed to improve the specific activity of the final product will be described and discussed. Attempts at further purification are also reported. The aim was to obtain a reconstituted proton pump with a specific ATPase activity comparable to that of the H<sup>+</sup>-ATPase purified by chromatographic methods.

### **3.2. METHODS.**

#### **3.2.1. Partial purification of the H<sup>+</sup>-ATPase.**

Chromaffin granule membranes were purified from fresh bovine adrenal medullae as described in the Methods section. They were stored at -20°C in 10 mM Hepes/NaOH pH 7, 1 mM DTT, 0.1 mM EDTA. After thawing, membranes were sedimented by centrifugation in a Beckman TL-100.3 rotor at 100,000 r.p.m. ( $g_{av}$  = 412,000) for 10 min. The membrane pellet was resuspended in working buffer (10 mM Hepes-NaOH or Tris-HCl/0.15 M KCl pH 7.4, 1mM DTT, 0.1 mM EDTA). Triton X-114 was added so that the final concentration of detergent was 11.5 mg/ml and protein concentration was 4 mg/ml. The mixture was homogenized and left on ice for 5 min. after which the pellet (P1) was removed by centrifugation as described above and washed by homogenization in the same buffer without Triton X-114, followed by centrifugation under the same conditions.

The white precipitate obtained was resuspended either in half the original volume of buffer, containing 18 mg/ml. of n-octyl- $\beta$ -glucoside (P1.a) or in the same original volume of buffer containing 15 mg/ml of the same detergent (P1.b). After a 10 min. incubation on ice, the mixture was centrifuged and the clear supernatant was carefully removed. This was the solubilized partially-purified ATPase.

#### **3.2.2. Stabilization of the partially purified ATPase.**

The enzyme solubilized in n-octyl- $\beta$ -glucoside had poor stability, losing 50% of its activity overnight, so in order to proceed with further purification, this material had to be stabilized. Several compounds were added to the solubilized ATPase, viz., 20% (w/v) solutions of sucrose, ethylene glycol, glycerol, ethanol and methanol mixed with equal volumes of solubilized enzyme. Methanol proved to be the only substance effective at stabilizing the protein under these conditions but was ineffective when added pure to the solubilized enzyme to the same final concentration (10%).

In order to achieve the stabilization of the enzyme without dilution, 1 ml bed volume Bio-Gel P6-DG columns, equilibrated with different detergents and 10% (w/v) methanol in working buffer, were poured in microfuge tubes, each with a glass wool plug and a pin-hole in the bottom, and mounted in the top of a conical centrifuge tube. Columns were packed by centrifugation at 1,400 r.p.m. for two or three minutes in a bench centrifuge and 200  $\mu$ l of solubilized ATPase were run through, also by centrifugation, and collected. This material collected from the Bio-Gel columns proved to be stable for several days (see Results).



### 3.2.3. Further purification of the H<sup>+</sup>-ATPase.

Two techniques were used:

#### 3.2.3.1. Gel filtration in Superose 12.

Pharmacia FPLC equipment was used. The Superose column was equilibrated with 0.1% C<sub>12</sub>E<sub>9</sub> in working buffer and 500 µl of the stabilized ATPase were injected. Forty samples of 0.5 ml were collected and assayed for ATP hydrolysis.

#### 3.2.3.2. Ultracentrifugation on glycerol gradients.

Glycerol gradients were formed as indicated in the Methods section. Initial experiments were carried out using 11 ml. gradients with a glycerol concentration range of 10 to 30%. Gradients were made in working buffer containing 10% (w/v) methanol, 0.1% (w/v) C<sub>12</sub>E<sub>9</sub> or MEGA-8. About 500 µl of stabilized ATPase were loaded and gradients were centrifuged at 40,000 r.p.m. for 15 h. in a Beckman SW 41 rotor.

Subsequent experiments were carried out as follows: 400 µl of stabilized ATPase were loaded on 5 ml 0-15% glycerol gradients and centrifuged for 5 hours at 45,000 r.p.m. in a Beckman SW.50.1 rotor. In both cases 0.5 ml samples were collected from the gradients and assayed for ATPase activity.

### 3.3. RESULTS.

The purification of the ATPase is summarized in Table 3.1. The effect of methanol and different detergents on the stability of the solubilized enzyme is shown in Fig 3.1(a) and (b). As can be seen, the presence of methanol is crucial for the stabilization of the solubilized material.

It is important to note that the stabilization procedure produces an increase in ATPase activity of between 1.5 and 3 times when 0.1% MEGA-8 or C<sub>12</sub>E<sub>9</sub> replace the n-octyl-β-glucoside. This enhancement is bigger when the P1 is solubilized in half the initial volume of buffer. Overall, an 8-fold increase in specific activity is achieved with a remarkably high recovery in the two conditions. The stabilized material solubilized in half the initial volume was used for further purification due to its higher concentration which made it easier to work with.

The ATPase solution became turbid when run through the Bio-Gel column. When this turbid solution was subjected to centrifugation (15 min. at 100,000 r.p.m.) almost all the activity could be found in the pellet obtained. However, no H<sup>+</sup>-translocation was observed when this pellet was assayed for ACMA quenching (see Materials and Methods chapter).



	ATPase Activity (nmol/min/ml)	Protein Concentration (mg/ml.)	Specific Activity (nmol/min/mg)	Recovery ATPase-act. ( % )
<b>Membranes</b>	910	4	227	100
<b>Washed P1</b>	414	0.44	940	46
<b>Solubilized P1</b>				
- Half initial vol.	300	0.60	500	27.7
- Same initial vol.	331	0.25	1324	36.7
<b>Stabilized P1 (C<sub>12</sub>E<sub>9</sub>)</b>				
- Half initial vol.	910	0.60	1500	50
- Same initial vol.	500	0.25	2000	55.5

**Table 3.1. Typical table of purification of chromaffin granule ATPase by Triton X-114 fractionation.**



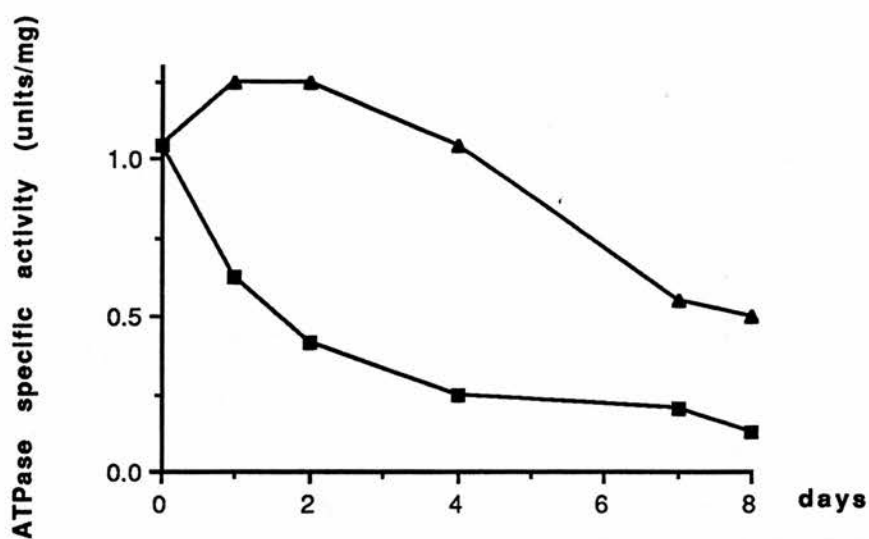


Figure 3.1.a. Stability of ATPase activity in the presence (▲) and absence (■) of methanol. Both samples are in n-octyl-β-glucoside.

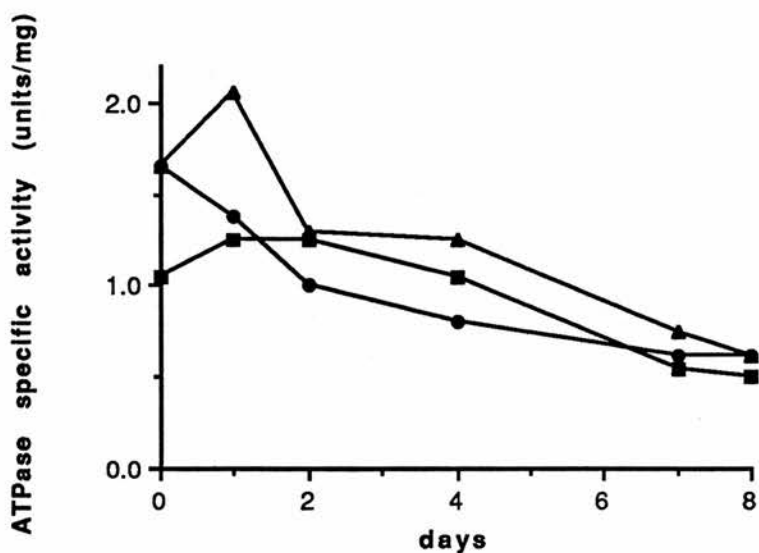


Figure 3.1.b. Effect of n-octyl-β-glucoside (■), C<sub>12</sub>E<sub>9</sub> (●) and MEGA-8 (▲) on the stability of the solubilized ATPase. 10% methanol is present in all cases.

### **3.3.2. Further purification of the ATPase.**

#### **3.3.2.1. Gel filtration in Superose 12.**

The peak of ATPase activity appeared in samples 5-6 (40 fractions were collected) coinciding with a shoulder in a peak of material absorbing at 280 nm (Fig. 3.2(b)). SDS-PAGE analysis of the first sixteen fractions collected from the gel filtration (Fig. 3.2(a)) revealed the presence of bands supposed to be the subunits of the H<sup>+</sup>-ATPase (120, 72, 57, 40, 33 and 16 kDa), along with other bands at 45, 43, 37, 18-19 and 10-12 kDa. However, some subunits appear in considerable amounts in all fractions. The most important point is the separation of DBH and cytochrome b<sub>561</sub> from the peak of ATPase activity. As far as ATPase activity is concerned, the material recovered from the gel filtration had a lower specific activity than the initial material injected.

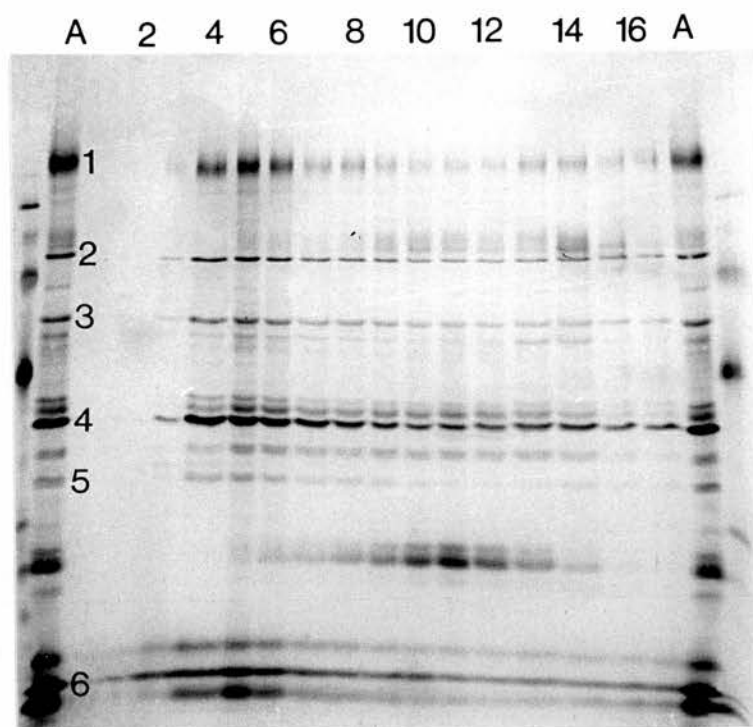
Fig. 3.3 shows a SDS-PAGE analysis of samples from the different stages of the purification from the chromaffin granule membrane.

After just one attempt, the apparatus became temporarily unavailable so attention was focused on glycerol gradients.

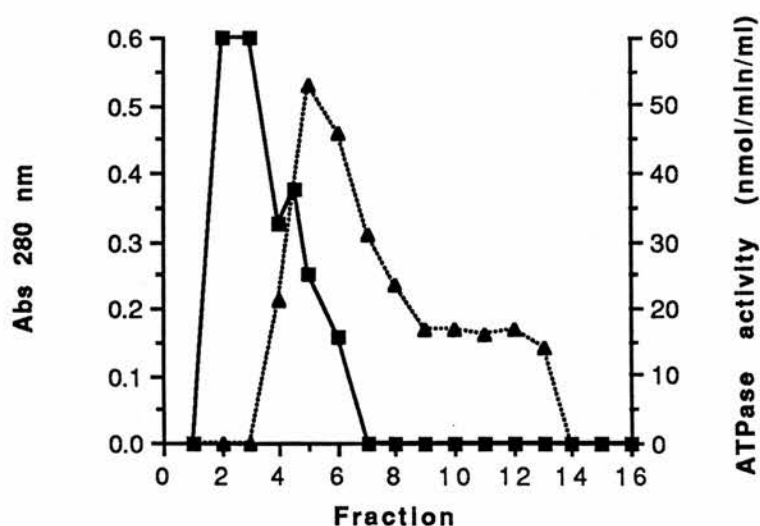
#### **3.3.2.2. Glycerol gradients.**

Initial attempts to centrifuge the stabilized ATPase through 10-30% glycerol gradients produced negative results, all the protein appearing at the top of the gradients after overnight centrifugation. It was then decided to try 0-15% gradients. In this case, activity was found in some samples (usually around the top middle of the gradients) and the recovery was very good. However, the specific activity was not improved. SDS-PAGE analysis of the samples recovered from the gradients and distribution of ATPase activity through the gradients are shown in Fig. 3.4(a) and (b) respectively. As in the gel filtration (see above), the bands corresponding to the different subunits of the enzyme appear along with many other bands and a quite strong background which suggests that little further purification has been achieved.

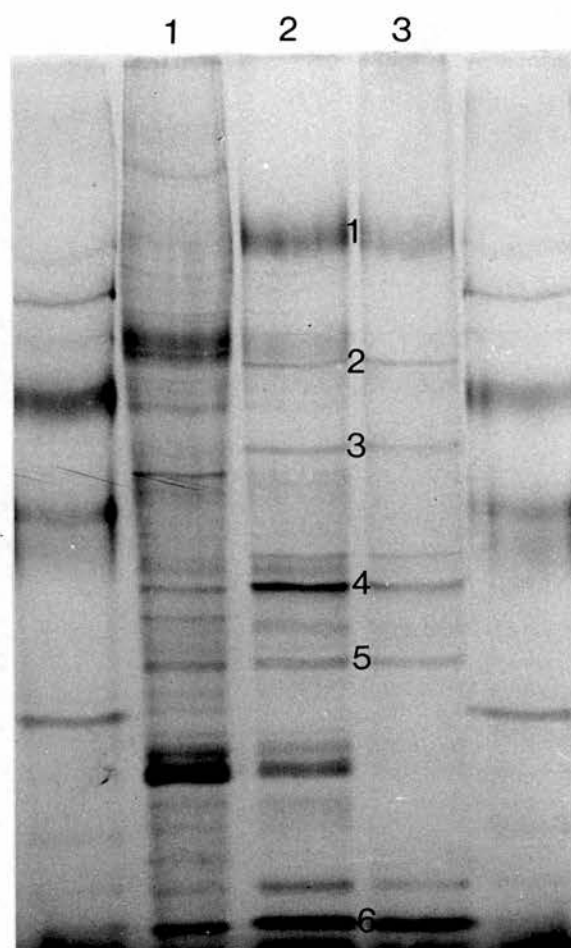
Interestingly, when the amount of Triton X-114 used initially to purify the enzyme was increased to 20 mg/ml. (instead of 11.5 mg/ml.) remarkably different results were obtained: first, the increase in ATPase activity produced during the stabilization of the solubilized enzyme was smaller (sometimes even non-existent), which means a lower specific activity at this stage and, second, when this material was loaded on glycerol gradients, further purification was achieved, as observed in Fig. 3.5. However, despite its purity as judged by gel electrophoresis, the activity was low and the inclusion of ATP or lipids such as PC or PS in the gradients made no difference.



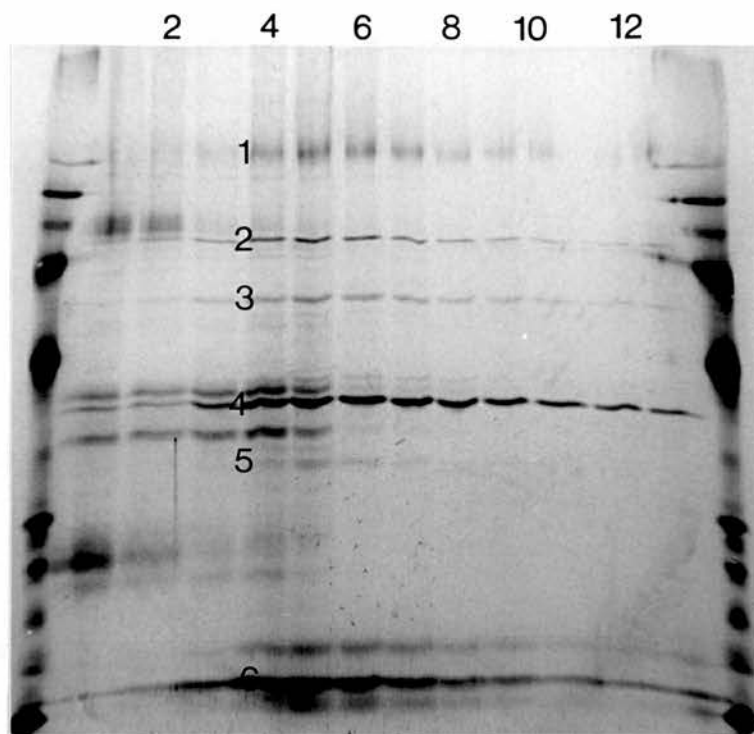
**Figure 3.2(a).** SDS-PAGE analysis of the first 16 fractions collected from the gel filtration in Superose 12. Tracks 1–16, samples from the column; A, loaded material (i.e., stabilized ATPase); outer tracks, molecular-mass standards [from top to bottom:  $\beta$ -galactosidase (116 kDa), phosphorylase b (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa)]. Tracks 1-16 contained 50  $\mu$ l of material each. Staining was with silver. The numbers to the left of track 1 indicate the putative ATPase subunits (120, 72, 57, 41, 33 and 16 kDa).



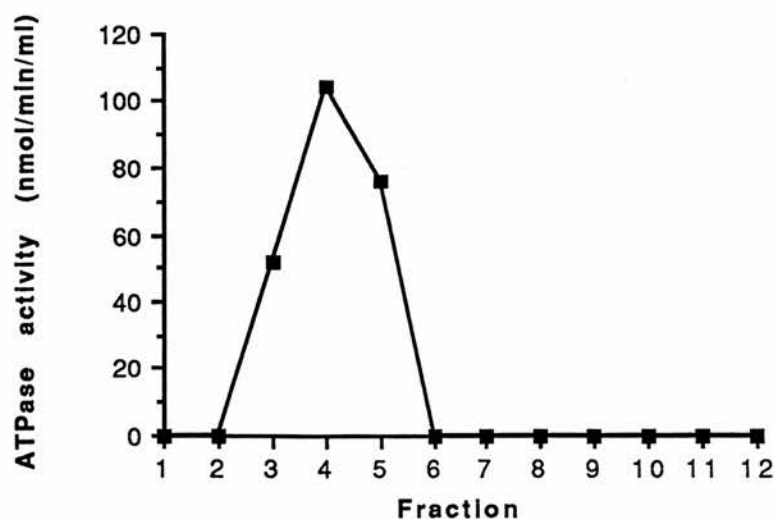
**Figure 3.2(b).** Distribution of protein (■—■) and ATPase activities (▲.....▲) in the first 16 samples collected from gel filtration in Superose 12.



**Figure 3.3. SDS-PAGE analysis of samples taken at different stages of the purification procedure.** Track 1, purified chromaffin granule membranes; track 2, stabilized ATPase; track 3, pool of fractions 4, 5 and 6 from the gel filtration. Outer tracks, molecular mass standards as on Figure 3.2(a). Staining was with silver. Numbers between tracks 2 and 3 indicate putative ATPase subunits -see Figure 3.2(a)-.

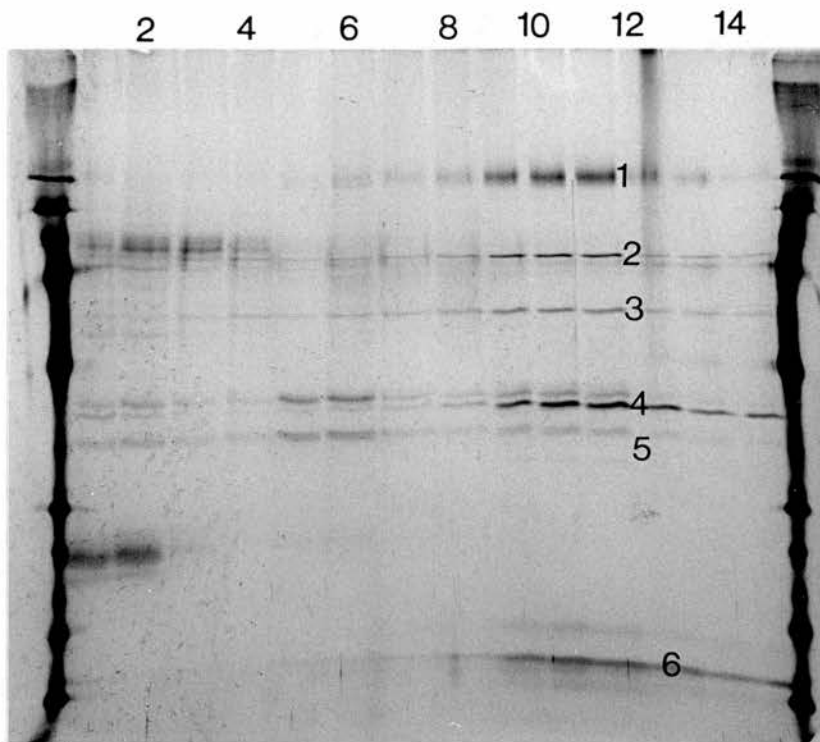


**Figure 3.4(a). SDS-PAGE analysis of fractions collected from glycerol gradients.** Tracks 1-12, fractions collected after ultracentrifugation of stabilized ATPase on 0-15% glycerol gradients. Fraction 1 is top of the gradient and fraction 12 bottom. About 100  $\mu$ l of sample were loaded in each track. Stain was with silver. Outer tracks, molecular mass standards as in Figure 3.2(a). ATPase subunits are highlighted with numbers between track 3 and 4. In this case, stabilized ATPase purified with 11.5 mg/ml of Triton X-114 was loaded onto the gradients. See text for further details.



**Figure 3.4(b). Distribution of ATPase activity in the fractions collected from the glycerol gradient shown in figure 3.4(a).**





**Figure 3.5. SDS-PAGE analysis of fractions collected from glycerol gradients.** Tracks 1-14, fractions collected after ultracentrifugation of stabilized ATPase on 0-15% glycerol gradient. Track 1 is top and track 14 is bottom of the gradient respectively. As in figure 3.4(a), about 100  $\mu$ l of each sample were loaded per track and staining was with silver. Outer tracks are molecular mass standards as in figure 3.2(a). Putative chromaffin granule ATPase subunits are highlighted with numbers on the right of track 11: (1) 120 kDa, (2) 72 kDa, (3) 57 kDa, (4) 40 kDa, (5) 33 kDa, (6) 16 kDa. In this case, stabilized ATPase was purified with 20 mg/ml Triton X-114. Only residual ATPase activity could be found in fractions 9, 10 and 11.

### 3.4. DISCUSSION.

The initial aim of adapting the Triton X-114 fractionation in order to obtain a product with a reasonably good specific activity was achieved to some extent. The partially purified ATPase obtained was quite stable and the recovery was remarkably high. However, this material proved to be difficult to purify any further by gel filtration or density gradient centrifugation. Nevertheless, important conclusions could be drawn from these unsuccessful attempts.

The fact that the activity of the solubilized ATPase was improved when run through the Bio-Gel columns equilibrated with small concentrations of detergents suggested that mixed micelles containing proteins, detergent and intrinsic lipids are formed. These vesicles could be precipitated by centrifugation. In these aggregates n-octyl- $\beta$ -glucoside is replaced by other detergents allowing the interaction between proteins and lipids that are present in what we call P1. P1 constitutes the hydrophobic fraction of the chromaffin granule membrane, which means that it contains the most hydrophobic proteins and a large fraction of the membrane lipids (Pryde and Phillips, 1986).

When the stabilized ATPase was subjected to gel filtration, vesicles containing the mixture of proteins present in the P1 were eluted quite rapidly, being separated only from a very small fraction of proteins that remain in solution after the removal of detergent, normally DBH and cytochrome  $b_{561}$  which are less hydrophobic than the rest of proteins contained in the P1. Actually, in the Triton X-114 fractionation, the bulk of these two proteins appear in the detergent-rich fraction (Pryde and Phillips, 1986). Overall, little purification was achieved.

As far as glycerol gradients are concerned, 10-30% gradients proved useless because the aggregates floated. When 0-15% glycerol gradients were used, the same problem as with gel filtration appeared: protein/lipids aggregates were only separated from proteins remaining in true solution. However, when the initial amount of Triton X-114 used to fractionate the chromaffin granule membranes was increased to 20 mg/ml, the amount of lipid present in the P1 fraction was presumably lower than in the previous case. When the aggregates thus obtained were loaded onto the glycerol gradients, they were solubilized by the detergent present in the gradients and a good separation was achieved. The lower activity showed by the protein in this case was probably due to a lowered amount of lipid.

The fact that the clean enzyme obtained from the glycerol gradients had a poor activity whereas the cruder, protein-lipid aggregates did not lose any activity suggested that the presence of lipids was crucial for the enzyme to maintain its optimal conformation. However, the addition of lipids to the gradients did not improve the activity of the purified enzyme, which further suggested that removal of detergent was needed in order to allow the interaction between protein and lipids, the dilution produced in the activity assay not being an effective means of promoting

this interaction.

As far as the role of the detergents is concerned, the fact that substitution of n-octyl- $\beta$ -glucoside by smaller amounts of Mega-8 and C<sub>12</sub>E<sub>9</sub> improved the enzymic activity when intrinsic lipids were present can be explained in two different ways: either the hydrophobic chains of these detergents are able to interact with the proteins, acting as substitutes of lipid molecules, or they improve and stabilize lipid-protein interactions. In both cases, the molecules of detergent would be part of the aggregates and, presumably, these structures would be leaky, preventing the accumulation of protons into them by the enzyme. This would explain why no proton translocation was observed using the ACMA assay.

Finally, we can conclude that, although a satisfactory method of purification was not achieved, the experiments described above have helped us to understand the behaviour of the chromaffin granule H<sup>+</sup>-ATPase with respect to lipids and detergents. The conclusions drawn from this chapter permitted the design of new strategies in order to achieve the aim of the purification and characterization of the H<sup>+</sup>-ATPase. These strategies will be discussed in the following chapters.

## **Chapter 4**

### **RECONSTITUTION OF THE CHROMAFFIN GRANULE H<sup>+</sup>-ATPase**

#### 4.1. INTRODUCTION.

It has already been mentioned that several different methods of purification for the chromaffin granule ATPase can be found in the literature. In some of them (Cidon and Nelson, 1986; Percy *et al.*, 1985) no reconstitution into proteoliposomes was attempted. In the method of Cidon and Nelson a very high specific activity (17 units/mg) was reported, however, as expected, no H<sup>+</sup>-translocation could be assayed. Furthermore, the high specific activity of this preparation came from the division of a very low activity by a minute protein concentration. This implies that the results might not be very accurate and also that the final material was not suitable for further studies due to this very low activity.

In 1987, Moriyama and Nelson published a new method in which reconstitution was carried out. In this case, it was achieved by the dilution of the peak fractions obtained from glycerol gradients; these contained the purified H<sup>+</sup>-ATPase along with intrinsic lipids from the chromaffin granule membrane. Obviously, the composition of the liposomes thus obtained was unknown.

Reconstitution into liposomes made with defined mixtures of commercial lipids has been reported with H<sup>+</sup>-ATPase of clathrin-coated vesicles: in a study published in 1986, Xie *et al.* carried out exhaustive studies on the role of different phospholipids in supporting ATP-dependent H<sup>+</sup>-translocation by the enzyme. As a result, an optimal mixture of phospholipids and cholesterol was defined for this particular protein.

In the previous chapter, studies on the purification of the chromaffin granule H<sup>+</sup>-ATPase were discussed. It was concluded that reconstitution of the enzyme could be achieved by solubilizing the hydrophobic fraction of the chromaffin granule membrane in a nonionic, mild detergent, of low micelle size and high critical micelle concentration, such as n-octyl- $\beta$ -glucoside, and removing the latter by gel filtration. However, the liposomes thus obtained were unable to support a measurable transmembrane gradient of protons and, moreover, their lipid composition was undefined. This material was also contaminated with DBH and cytochrome b<sub>561</sub>, the major proteins in chromaffin granule membranes.

In the present chapter, I discuss the development of a new method of purification and reconstitution for the chromaffin granule H<sup>+</sup>-ATPase. The method of purification is basically the same as the one reported previously with further steps aimed at the delipidation of the partially purified enzyme and addition of commercial lipids to this material. The objective was to study the effect of different lipids on the enzymic activities in order to obtain a lipid mixture that optimized both H<sup>+</sup>-translocation and ATP hydrolysis. The delipidation of the solubilized ATPase was important in order to ensure that the effects observed with commercial lipids were not biased by the presence of intrinsic lipids.

The final result was a method that yields reconstituted, partially purified chromaffin granule H<sup>+</sup>-ATPase in a quite stable form. The procedure can be accomplished in two hours, and the specific ATPase activity is comparable to that reported by other groups.



## **4.2. METHODS.**

### **4.2.1. Purification of the H<sup>+</sup>-ATPase.**

Chromaffin granule membranes were purified from fresh bovine adrenal medullae as described in the Methods section, and stored at -20°C in 10 mM Hepes/NaOH, pH 7.4, 0.1 mM EDTA, 1 mM DTT. After thawing, membranes were sedimented by centrifugation in a Beckman TL100.3 rotor at 100,000 r.p.m. ( $g_{av}$  = 412,000) for 10 min. The membrane pellet was resuspended in working buffer (10 mM Hepes-NaOH or Tris-HCl/0.15 M KCl, pH 7.4, 0.1 mM EDTA, 1 mM DTT). Triton X-114 was added so that the final concentrations were: detergent 20 mg/ml, protein 4 mg/ml. The mixture was homogenized in a Teflon/glass homogenizer and left on ice for 5 min. after which, the pellet was removed by centrifugation as described above and washed by homogenization in the same buffer, containing 20 mg/ml Triton X-114, followed by centrifugation. The white precipitate obtained was resuspended in half the original volume of buffer, containing 18 mg/ml n-octyl- $\beta$ -glucoside. After a 10 min incubation on ice, the mixture was centrifuged and the clear supernatant was carefully removed. This was the solubilized, partially purified ATPase.

### **4.2.2. Reconstitution into phospholipid vesicles.**

Pure lipids [solutions in chloroform/methanol (2:1, v/v)] were dispensed into glass tubes in the required amounts and dried under a stream of nitrogen, 200  $\mu$ l aliquots of solubilized ATPase were added and the lipids dispersed using a stirring rod. The mixture was then transferred to a small glass/glass homogenizer (0.5 ml) using a Pasteur pipette, and homogenized gently. Each solution was incubated at room temperature for about 10 min. and loaded onto a 1 ml column of Bio-Gel P6-DG that had been equilibrated with 10% (v/v) methanol in working buffer and packed by centrifugation in a bench centrifuge (1 min, 1400 r.p.m./150  $g_{av}$ ). The detergent-lipid-protein mixtures were then passed through the column by centrifugation under the same conditions. Normally, white cloudy solutions were collected from the columns which suggested the formation of vesicles.

The reconstituted ATPase was usually kept on ice for at least 30 minutes before being subjected to activity assays (H<sup>+</sup>-translocation and ATP hydrolysis) although no difference could be observed if the assays were carried out just after the purification-reconstitution procedure. Subsequently, samples were kept in a refrigerator at 4 °C and were used for further studies (see next Chapter) during several days.

## 4.3. RESULTS.

### 4.3.1. Purification of the H<sup>+</sup>-ATPase.

The purification method is summarized in Table 4.1. It can be observed that a considerable loss of activity occurs in the second step (washing of the initial pellet). However, this step is crucial for several reasons: (1) it eliminates completely ATPase II from the pellet, (2) it further depletes the pellet of two major contaminant proteins: cytochrome b<sub>561</sub> and DBH, (3) it minimizes the contamination of endogenous lipids ensuring that the effects observed during reconstitution are due to the added pure lipids and not to the endogenous ones (see Fig. 4.1). The mixture used for reconstitution in the experiments shown in the Table was: 34.2% PC, 34.2% PE, 11.1% PS and 20.6% cholesterol. The lipid to protein ratio varied between 10-30 µg lipids/µg protein. Fig 4.2 shows a SDS/polyacrylamide gel with samples from the different stages of the purification. The reconstituted enzyme contains the six major polypeptides of 120, 72, 57, 40, 33 and 16 kDa that have been assigned to the ATPase (Percy *et al.*, 1985; Moriyama and Nelson, 1987) and contaminants of 78, 38 and 36 kDa. It is noteworthy that the reconstitution step is quite selective, the detergent n-octyl-β-glucoside specifically solubilizing the ATPase from the mixture of proteins in the pellet.

The reconstituted ATPase had good stability, losing between 4-10% of the activity in 24 hours at 4 °C. However, the solubilized ATPase lost activity quite rapidly and it was important to proceed at once with the reconstitution. The presence of methanol also proved to be crucial for stabilizing the enzyme (see previous chapter). KCl provides osmotic support for the formation of vesicles and it can be replaced by 0.3 M sucrose although in this case, the vesicles were less stable.

### 4.3.2. Reconstitution of the H<sup>+</sup>-ATPase.

#### 4.3.2.1. Measurement of proton-translocation using fluorescent probes: the ACMA quenching assay.

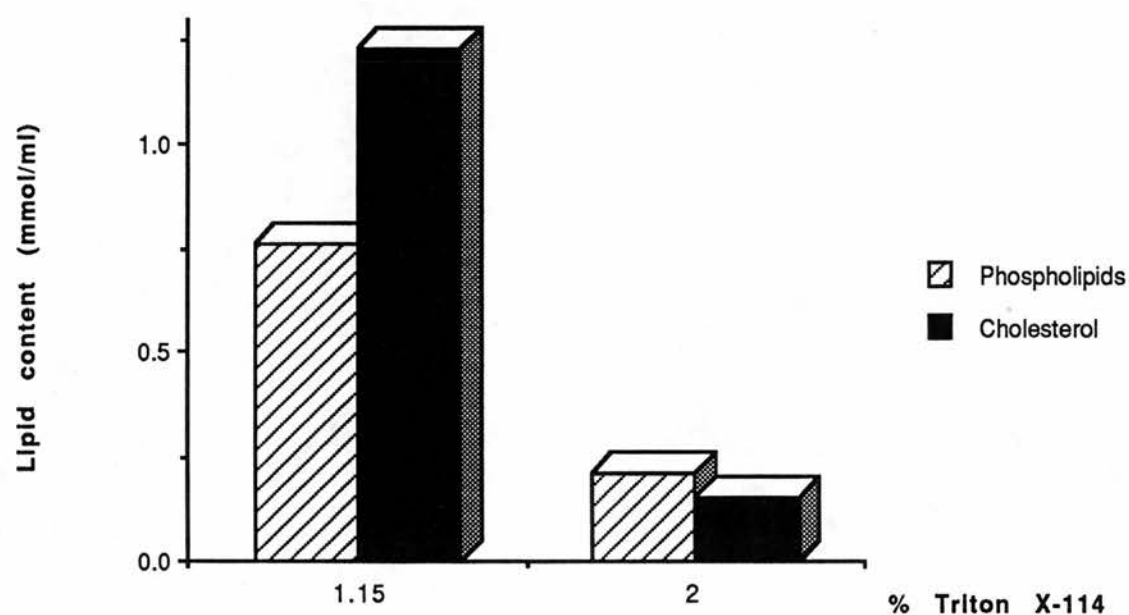
The distribution of a weak base across the membrane of a subcellular compartment is related to the concentration of protons outside and inside the compartment. In the case of monoamines with a high pK<sub>a</sub> and assuming that the uncharged amine is freely permeant and is the only form that crosses the membrane, the expression that links amine and proton distributions across a biological membrane is:

$$[A]_{in} / [A]_{out} = [H]_{in} / [H]_{out}$$

Stage	ATPase activity (nmol/min/ml)	[Protein] (mg/ml)	Specific activ. (nmol/min/mg)	Recovery ( % )
Membranes	2815	7.6	370 (348±26)	100
Precipitate	1546	1.4	1140 (1263±222)	90.0 (87.7±6.9)
Washed ppte.	711	1.0	711 (1120±355)	19.7 (26.1±4.3)
Solubilised ATPase	200	0.21	952 (894±195)	6.8 (7.0±1.8)
Reconstituted ATPase	500	0.21	2380 (2453±139)	15.1 (16.6±1.9)

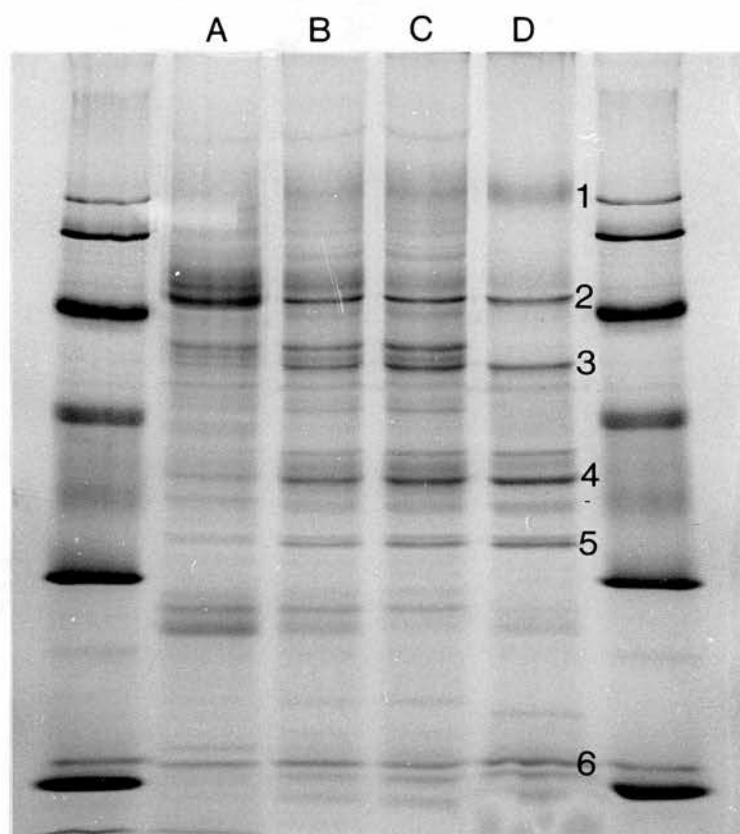
**Table 4.1. Reconstitution of chromaffin granule ATPase I.**

The data shown are for a typical preparation; values in parentheses are the means  $\pm$  standard deviations of specific activities and recoveries obtained in four separate preparations. Recovery values are referred to ATPase activity.



**Figure 4.1. Lipid content in the solubilized ATPase after treatment of chromaffin granule membranes with different amounts of Triton X-114.**

Membranes were fractionated and the pellet obtained washed with the indicated amounts of Triton X-114. Solubilization was performed as specified in text. Lipids were estimated as indicated on the Materials and Methods section.



**Figure 4.2. SDS-PAGE analysis of ATPase fractions.**

Track A, chromaffin granule membranes; track B, protein precipitated with Triton X-114; track C, washed precipitate; track D, reconstituted ATPase; outer tracks, molecular-mass standards [from top to bottom:  $\beta$ -galactosidase (116 kDa), phosphorylase b (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa)]. Tracks A-D contained 50  $\mu$ g of protein each. Staining was with Coomassie Blue. The numbers to the right of track D indicate the ATPase subunits (120, 72, 57, 41, 33 and 16 kDa).

In the case of a fluorescent aminoacridine derivative, accumulation of protons inside a sealed vesicle drives the accumulation of the amine resulting in quenching of its fluorescence: an overall fall in the fluorescence of the solution is recorded. The extent of this quenching can be related to the  $\Delta\text{pH}$  across the membrane according to the expression (Schuldiner *et al.*, 1972):

$$\log [Q/1-Q] = \text{pH}_{\text{out}} - \text{pH}_{\text{in}} + \log V$$

Here  $Q$  is the fraction of the total fluorescence that is quenched and  $V$  the fractional volume of the osmotic compartment, relative to the total volume of the solution.

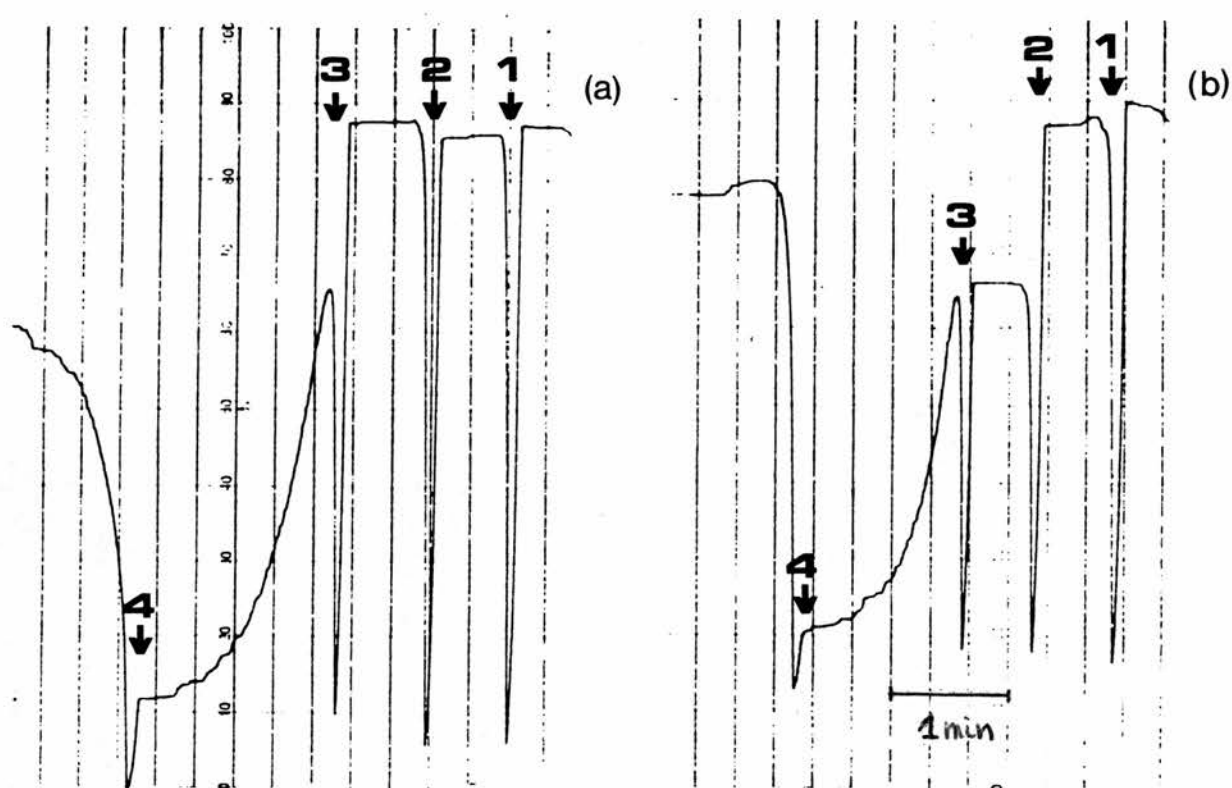
ACMA or 9-amino-6-chloro-2-methoxyacridine is a fluorescent acridine derivative that has been utilized to measure  $\Delta\text{pH}$  in intact chromaffin granules (Haigh *et al.*, 1989) and to measure  $\text{H}^+$ -translocation into resealed "ghosts" (Percy *et al.*, 1985). We have used this assay in order to measure  $\text{H}^+$ -translocation for reconstituted chromaffin granule ATPase. Fig 4.3-4.5 show typical traces of fluorescence quenching obtained under different conditions.

In order to confirm that ACMA fluorescence quenching was a suitable method for measuring a pH difference across liposomal membranes, a titration experiment was performed: 10  $\mu\text{l}$  of reconstituted ATPase were used to produce ATP-dependent quenching of ACMA quenching, and once the maximal quenching was achieved the external pH of the solution was changed by addition of small amounts of HCl, which produced small increments in fluorescence, as the extent of quenching ( $Q$ ) was reduced by the decrease of the external pH. When  $\log [Q/1-Q]$  was plotted against the external pH ( $\text{pH}_{\text{out}}$ ) a straight line resulted, with a slope value very close to 1.0 (see Fig 4.6). This implies that ACMA behaves as predicted by the Schuldiner equation (assuming the  $\text{pH}_{\text{in}}$  and  $V$  are constant— see above) and is therefore suitable for measuring a transmembrane proton gradient. ACMA quenching was absolutely dependent on added ATP (Fig. 4.3(a)),  $\text{Mg}^{2+}$  (Fig. 4.3(b)) and valinomycin (Fig. 4.4). The presence of vanadate, an inhibitor of ATPase II, made no difference (Fig. 4.5).

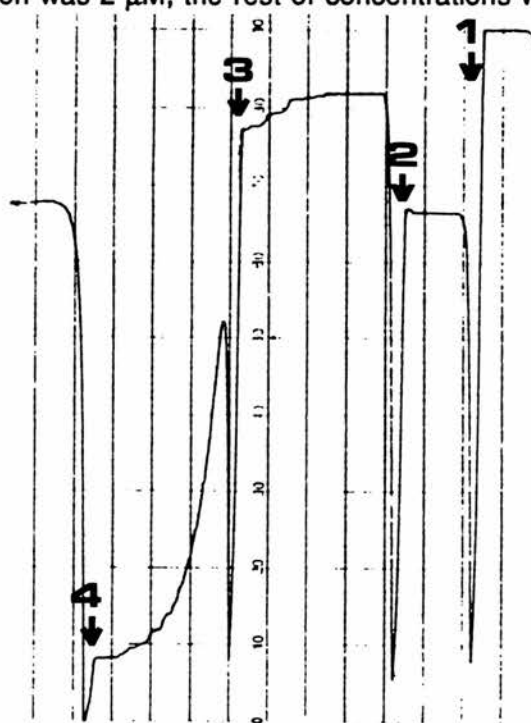
#### 4.3.2.2. Initial rate of fluorescence quenching as a measure of $\text{H}^+$ -translocation activity.

The use of the initial rate of fluorescence quenching as a measure of  $\text{H}^+$ -translocation activity is widely reported in the literature (Flatmark *et al.*, 1985; Gronberg and Flatmark, 1987; Gluck and Al-Awqati, 1984; Bennet and Spanswick, 1983; Thevenod *et al.*, 1989). Gluck and Al-Awqati (1983) and Bennet and Spanswick (1983) have produced theoretical justifications that this rate is actually proportional to the rate of proton accumulation into a sealed compartment. As far as the reconstituted chromaffin granule ATPase is concerned, the initial rate of ACMA quenching was shown to be proportional to the amount of protein present (Fig. 4.6) and it was decreased in parallel with the ATPase activity (fig. 4.7). As a consequence the initial rate of fluorescence quenching was taken as a direct measure of the  $\text{H}^+$ -translocation activity of the protein. A theoretical justification for this, which makes fewer assumptions than those published, appears in Appendix 2.

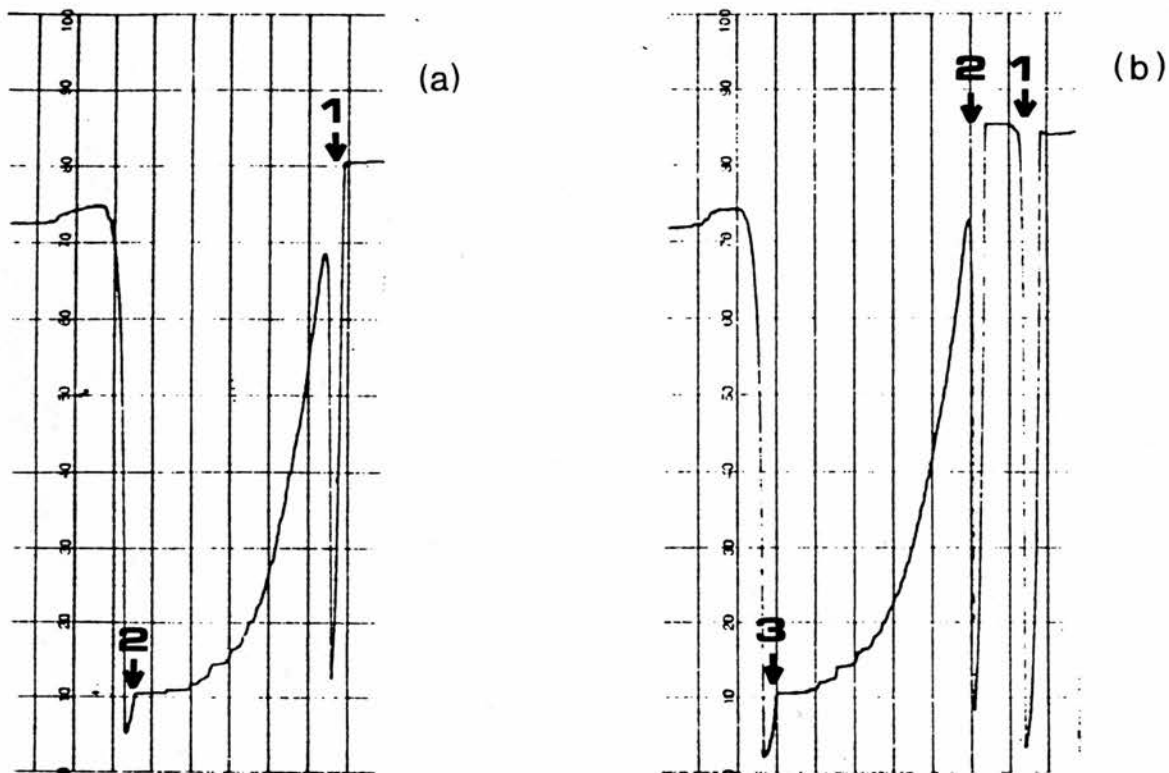




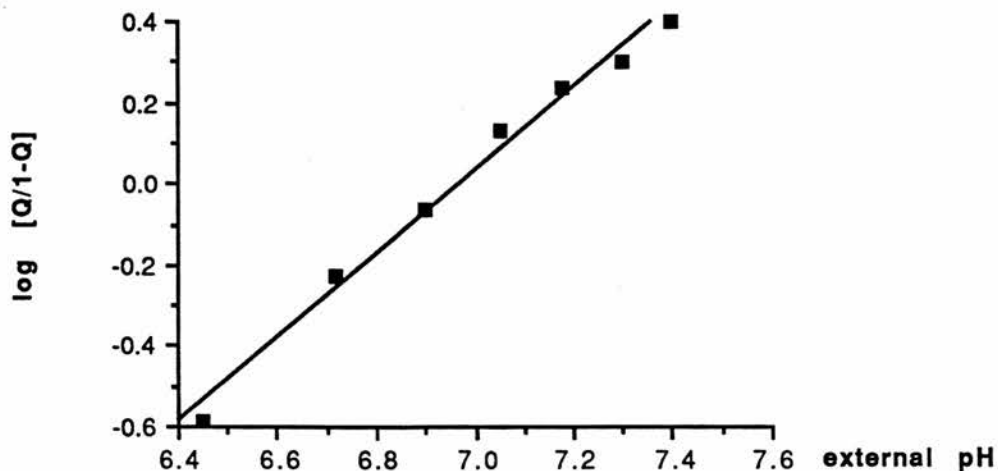
**Figure 4.3. Dependence of ACMA quenching on the addition of ATP (a) and  $Mg^{2+}$  (b).** 5  $\mu$ l of reconstituted ATPase (about 1  $\mu$ g) were incubated in assay medium (see Materials and Methods). In figure 4.3(a) where indicated valinomycin (1),  $MgSO_4$  (2), ATP (3) and nigericin (4) were added so that final concentrations were 0.4  $\mu$ g/ml, 1 mM, 1 mM and 10 ng/ml respectively. In figure 4.3(b) valinomycin (1), ATP (2),  $MgSO_4$  (3) and FCCP (4) were added. FCCP final concentration was 2  $\mu$ M, the rest of concentrations were as on Figure 4.3.



**Figure 4.4. Dependence of ACMA quenching on addition of valinomycin.** Where indicated ATP (1),  $MgSO_4$  (2), valinomycin (3) and FCCP (4) were added. Concentrations and assay conditions were as on Figure 4.3.

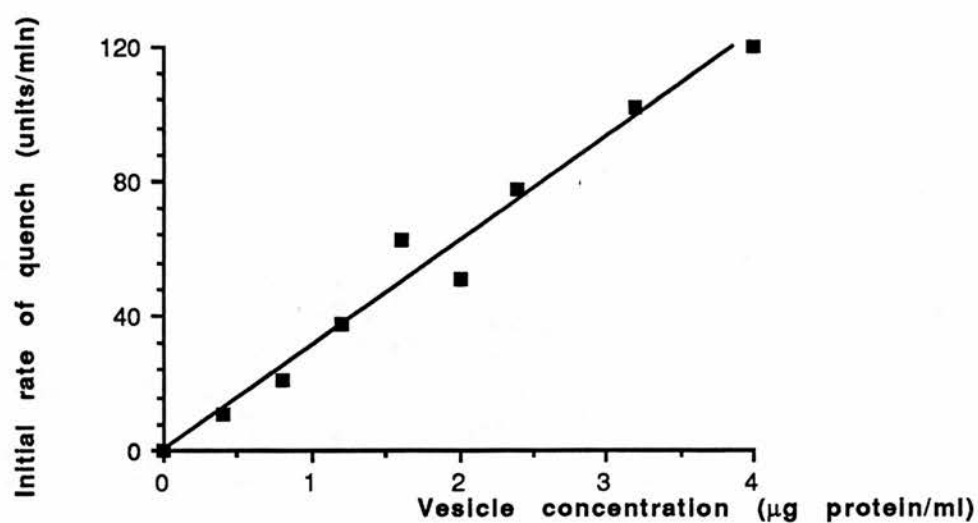


**Figure 4.5. Comparison of the rate of ACMA quenching in the absence (a) and presence (b) of vanadate.** MgATP (1) and FCCP (2)-fig. 4.6a- and vanadate (1), MgATP (2) and FCCP (3)- fig. 4.6b- were added to the medium containing 5  $\mu$ l reconstituted ATPase and 0.4  $\mu$ g/ml valinomycin. Final concentrations were : 2  $\mu$ M vanadate, 1 mM MgATP and 2  $\mu$ M FCCP.

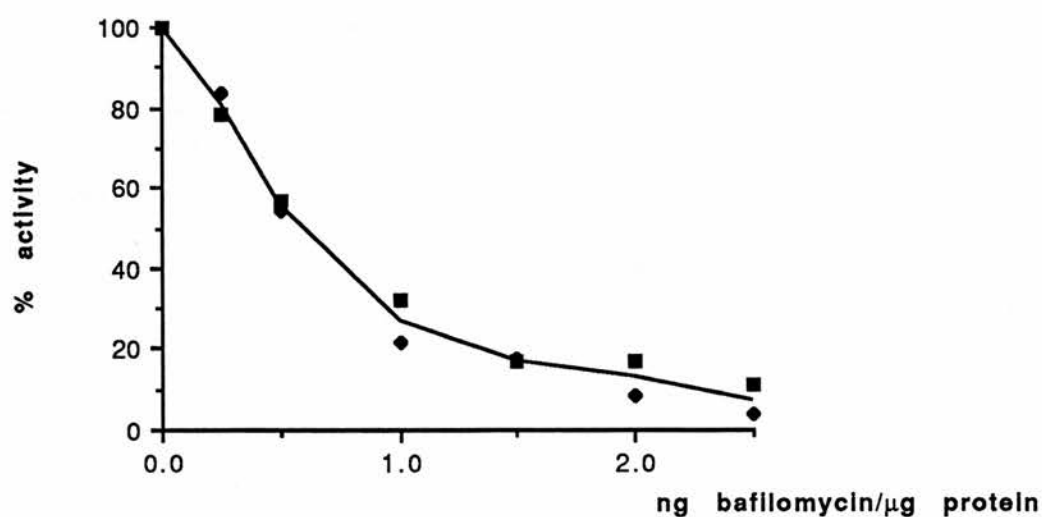


**Figure 4.6. Representation of external pH vs.  $\log [Q/1-Q]$  for ACMA quenching in reconstituted ATPase.**

The slope, calculated by least squares, is very close to 1.0 as predicted by the Schuldiner equation (see text for details)



**Figure 4.7.** Proportionality between the amount of protein and the initial rate of ACMA fluorescence quench.



**Fig 4.8.** Effect of Bafilomycin on ATPase activity (■) and initial rate of fluorescence quench (◆). Samples with different lipid to protein ratio were pooled and assayed for ATPase activity and ACMA assay as indicated in the Methods section. The lipid mixture utilized was the optimal one (see text).

#### 4.3.2.3. Effect of valinomycin on H<sup>+</sup>-translocation.

Valinomycin is a potassium-transporting ionophore which catalyses electrogenic transport of K<sup>+</sup> across lipid membranes. In this case, the concentration of K<sup>+</sup> inside the vesicles is 0.15 M whereas outside it is 3 mM from addition of vesicles in a KCl-containing medium. When the enzyme pumps protons into the sealed vesicles valinomycin prevents the formation of a transmembrane potential by permitting electrogenic leakage of K<sup>+</sup> ions out of the vesicles thus maintaining the charge balance across the membrane. In Fig 4.9 and 4.10 the effect of the presence of different amounts of valinomycin and KCl respectively on H<sup>+</sup>-translocation is shown.

#### 4.3.3. **Effects of lipids on ATPase and H<sup>+</sup>-translocation activities.**

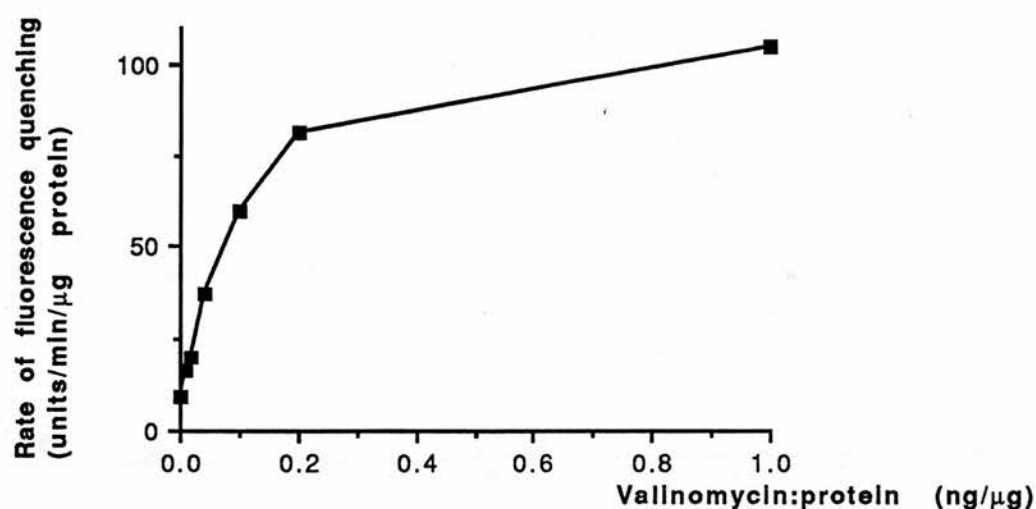
Several different sets of experiments were carried out in which the effects of different lipids were tested. The four lipids studied were: bovine spinal cord phosphatidylserine (PS), soybean phosphatidylcholine (PC), egg yolk and soybean phosphatidylethanolamine (PE) and cholesterol.

In the first set of experiments, PS and PC were tested by making vesicles with increasing amounts of one lipid and decreasing amounts of the other, maintaining a lipid to protein ratio of about 30 µg/µg of protein. Then the same amounts of PC and PS were tested in the presence of 3 and 6 µg cholesterol/µg of protein. The results are shown in Fig. 4.11(a) and (b): ATPase activity does not depend strongly on the lipid composition, although it is marginally higher when both lipids are present. Cholesterol does not have any significant effect. In contrast, H<sup>+</sup>-translocation is sharply dependent on the lipid composition, especially on the presence of cholesterol. In these experiments the optimal lipid mixture for generation of a measurable pH difference was PC:PS:cholesterol 20:10:6.

In the second set of experiments, PC was progressively replaced by PE in the presence of different amounts of cholesterol (0, 3 and 6 µg/µg of protein) maintaining a constant level of PS at 10 µg/µg of protein. The results of this are shown in figures 4.12(a) and (b). In this case, we observe again the requirement for cholesterol for H<sup>+</sup>-translocation and insignificant effects on ATPase activity. It can also be concluded from the figures that PC can be substituted by PE without any of the activities being much affected. From these results, the optimal lipid mixture found thus far was PC:PE:PS:cholesterol 10:10:10:6. No difference was observed between PE from soybean and egg yolk.

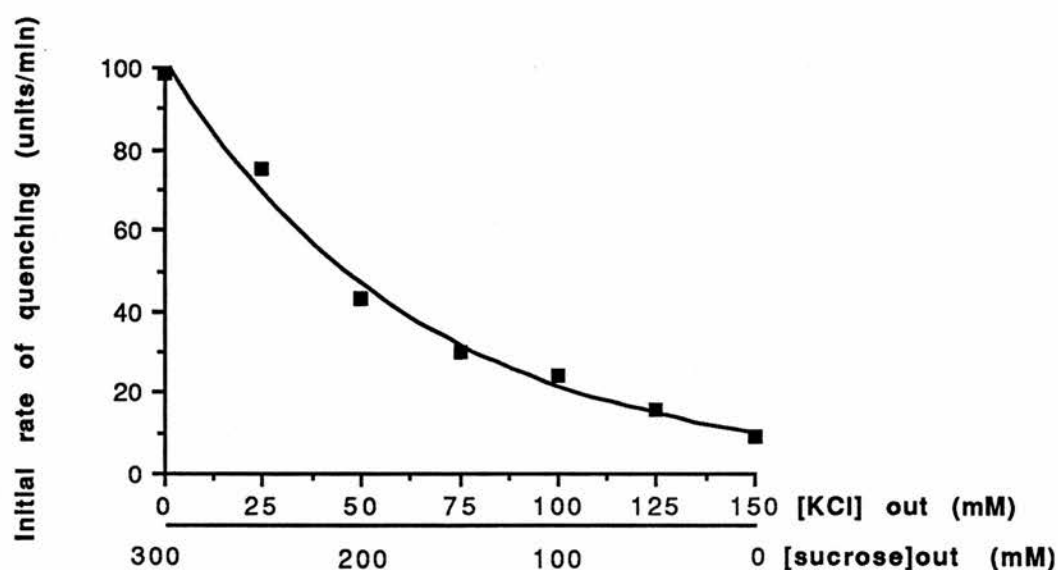
The role of PS was further studied in an experiment in which several lipid mixtures with different percentages of PS and the same ratio PC:PE:cholesterol 10:10:6, maintaining a lipid:protein ratio of about 36:1, were tested. The result is shown in Fig. 4.13.

From all the experiments carried out it was concluded that the optimal mixture of lipids for both activities was: 34.2% PC, 34.2% PE, 11.2% PS and 20.6% cholesterol.



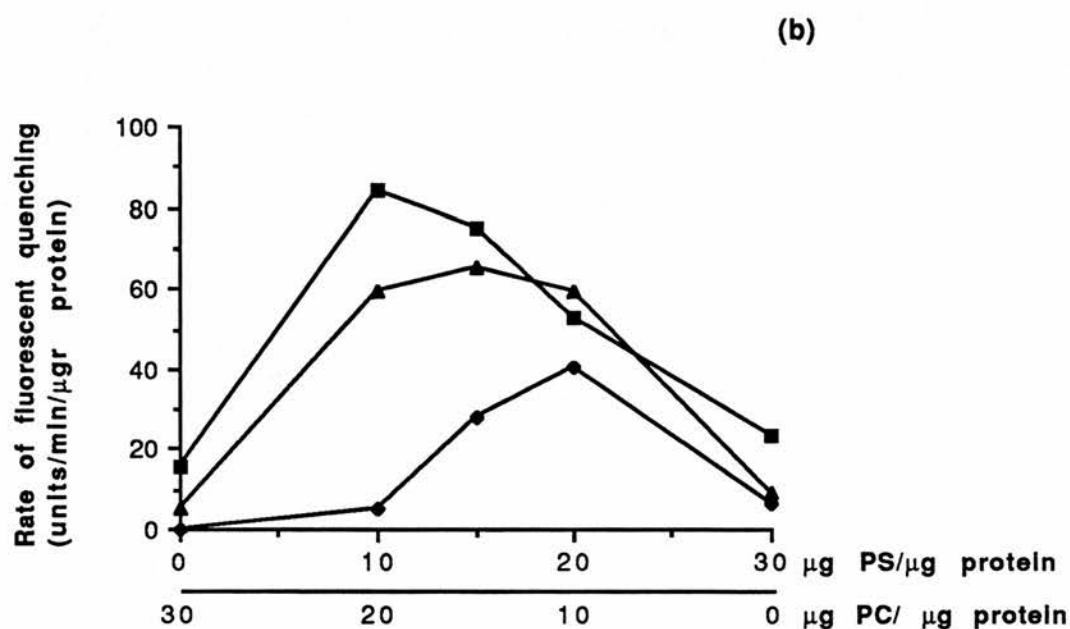
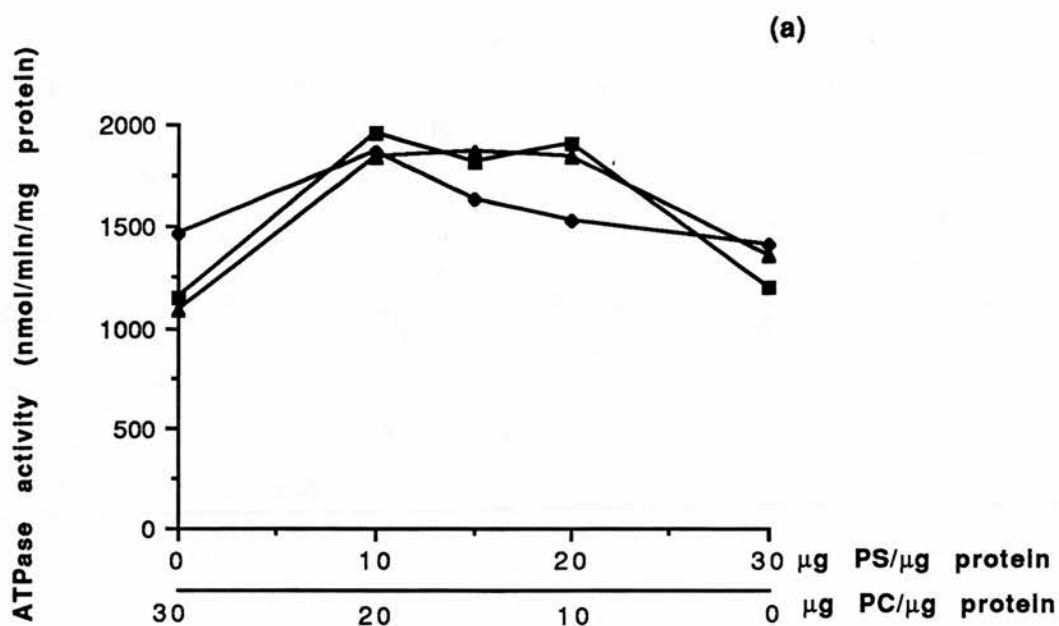
**Figure 4.9. Dependence of the initial rate of quenching of ACMA fluorescence on valinomycin concentration.**

The vesicle concentration was 4 μg of protein/ml and lipid composition was optimal (see text).



**Figure 4.10. Effect of the external concentration of KCl on the initial rate of quenching recorded.**

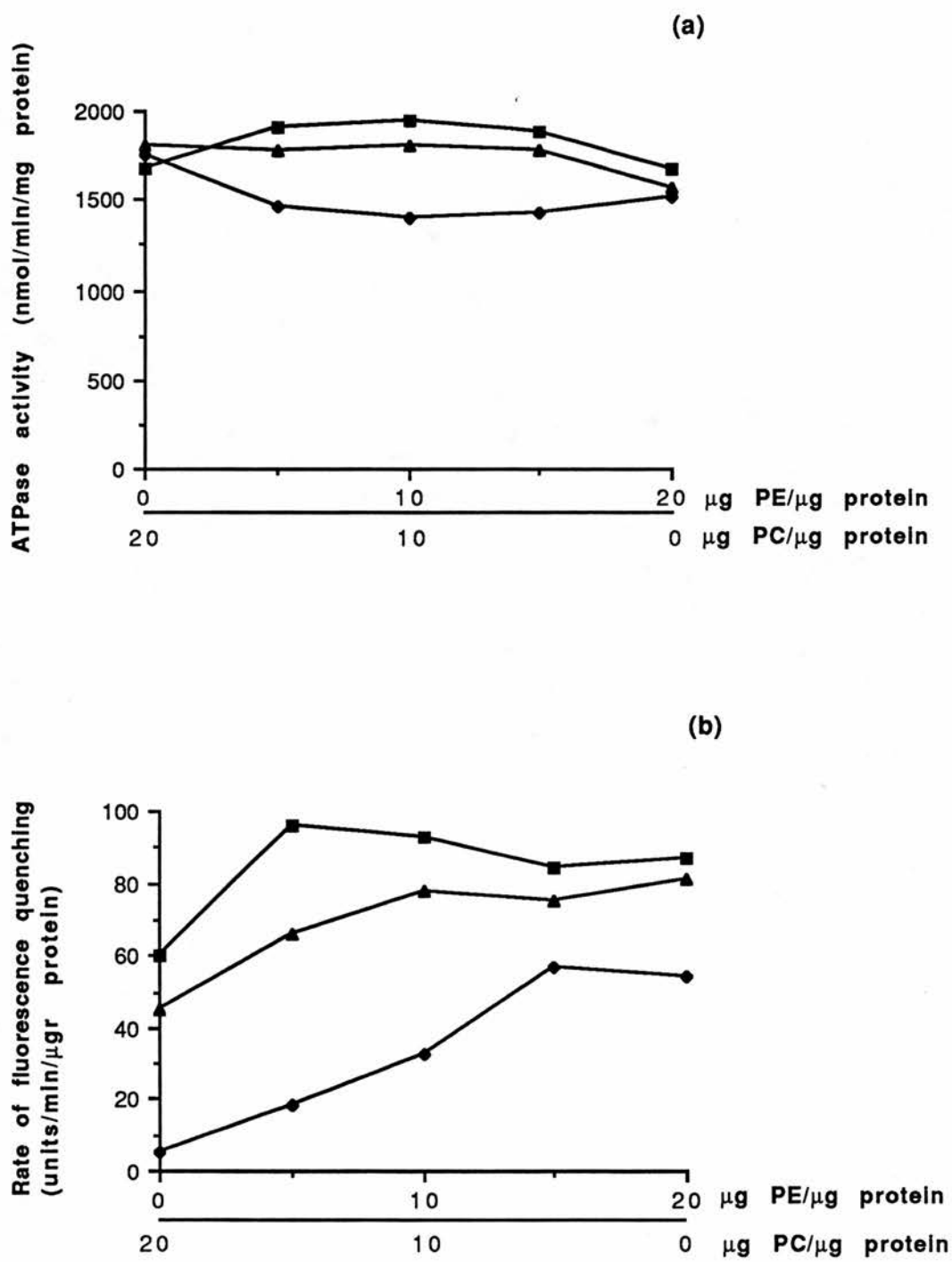
The amount of sucrose was accordingly varied so that the osmotic pressure remained constant in all cases.



**Figure 4.11. Dependence of ATPase activity (a) and ACMA fluorescence quenching rate (b) on the phosphatidylcholine/phosphatidylserine ratio.**

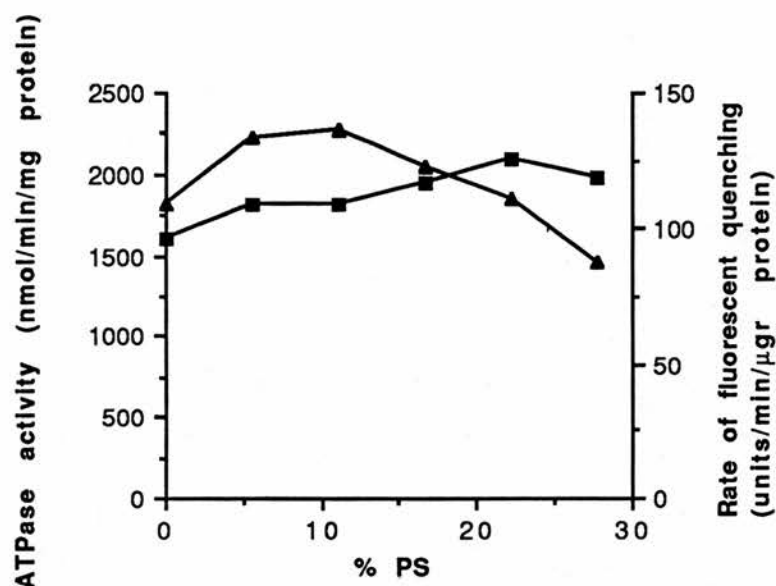
The total phospholipid content was 30  $\mu\text{g}/\mu\text{g}$  of protein and the cholesterol/protein ratio was 0 (◆), 3 (▲) or 6 (■)  $\mu\text{g}/\mu\text{g}$  of protein. The valinomycin concentration was 0.4  $\mu\text{g}/\text{ml}$ .





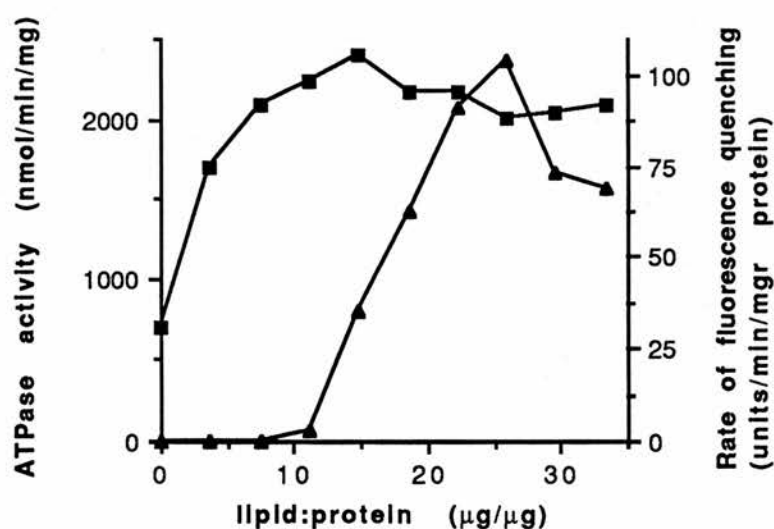
**Figure 4.12. Effect of replacing phosphatidylcholine by phosphatidylethanolamine.**

The phosphatidylserine content was constant at 10  $\mu\text{g}/\mu\text{g}$  of protein, and other conditions were as in Fig. 4.12.



**Figure 4.13.** Effect of the amount of PS present in the lipid mixture in ATP hydrolysis (■) and H<sup>+</sup>-translocation (▲).

The PC:PE:cholesterol ratio was maintained at 20:20:12, and the lipid to protein ratio was 36:1.



**Figure 4.14.** Dependence of ATPase activity (■) and ACMA fluorescence quenching rate (▲) on the lipid to protein ratio.

The lipid composition was the optimal (see text) and valinomycin 0.4 μg/ml was present.

Once the optimal lipid mixture was determined, an experiment was performed in which the dependence on both activities on the overall lipid to protein ratio was studied. The results of this experiment is shown in Fig. 4.14. It can be seen that ATPase activity reaches a maximum quite rapidly, at 10-15  $\mu\text{g}$  lipid/ $\mu\text{g}$  of protein, whereas  $\text{H}^+$ -translocation needs a higher ratio (25-30  $\mu\text{g}$  lipid/ $\mu\text{g}$  of protein). The presence of an excess of lipid seems to affect more the  $\text{H}^+$ -translocation than the ATPase activity.

#### 4.4. DISCUSSION.

The initial aim was to develop the previously-reported procedure of fractionating chromaffin granule membranes with Triton X-114 to give a product of specific activity comparable to that of the ATPase purified chromatographically. The new procedure can be completed in two hours and gives a product that is remarkably stable and free of ATPase II, as judged by its insensitivity to vanadate (see Results).

Moriyama and Nelson (1987) have reported another method for the purification of the chromaffin granule ATPase I that yields a product of higher specific activity (3-5 units/mg) but the reconstitution was achieved using endogenous lipids, thus the composition of the vesicles was undefined. Furthermore, the method involves a hydroxylapatite column and a glycerol gradient step, and is therefore considerably longer. This necessitates the use of protease inhibitors such as Leupeptin and Pepstatin A, not needed in our method.

The reconstitution into sealed vesicles able to support a measurable transmembrane pH gradient allows the use of fluorescent probes for assaying the activity of the enzyme. ACMA, as discussed in the Results section, follows the Schuldiner equation for permeant monoamines which confirms its suitability to measure a proton gradient across the vesicle membranes.

Very recently studies performed on the fluorescent probe acridine orange have cast doubt on the suitability of this type of method for measuring a transmembrane pH gradient (Palmgren, 1991). The main point of controversy is the ability of acridine orange and other acridine derivatives to form oligomers in the presence of anions which causes changes in its absorption spectrum thus amplifying the recorded quenching and making the accurate estimation of  $\Delta\text{pH}$  difficult. It is known that apparent values of osmotic volumes ( $V$ ) calculated from the Schuldiner equation (at  $\Delta\text{pH}=0$ ) are much higher than those obtained by more direct methods (Casadio, 1991) which suggests that fluorescent probes actually deviate from the ideal behaviour, that is, these compounds do interact with the membranes, moreover, this deviation between  $V$  values obtained from the Schuldiner equation and the values obtained by direct methods has always been considered as a measure of the "non-ideality" of the probes. However, Casadio (1991) taking into consideration hydrophobic interactions between ACMA and biological membranes has developed a transformed version of the Schuldiner equation that fits much better to the experimental data, although this version does not deviate significantly from the latter in the range of fluorescent quenching we are dealing with.

In any case, our objective is not measuring a  $\Delta pH$  but rather the speed at which this gradient is created by the enzyme. Also, Marty *et al.* (1986) have shown that ACMA does not dimerize in solution at concentrations as high as 200  $\mu M$ . The consequence of all this is that ACMA in particular seems not to present the problems that acridine orange reportedly has, at least in the conditions under which we use it. A good proof that ACMA does measure the speed of formation of a transmembrane proton gradient is figure 4.8 in which a vacuolar ATPase specific inhibitor such as Bafilomycin A<sub>1</sub> inhibits in parallel both ATPase activity and the rate of initial fluorescence quenching. The proportionality between the initial rate of quenching and the amount of protein present in the assay is a further indication that the former reflects the rate of proton-translocation and therefore is a valid assay of enzyme activity. This approach has also been used by other groups in the literature and justified on theoretical grounds (see above).

The role of FCCP, a proton ionophore that equilibrates the proton concentration across biological membranes, and nigericin, that exchanges protons and potassium ions across membranes, restoring the fluorescence to its initial value (see fig. 4.3-4.5) demonstrates the link between quenching and proton gradient. Also notable is the very rapid return of the recorded fluorescence to basal values on addition of these ionophores, confirming that the response of the probe is not limiting in the measurement of generation of  $\Delta pH$ . The fact that the occurrence of this proton gradient is absolutely dependent on ATP and  $Mg^{2+}$  further supports the role of the ATPase I as the creator of that gradient.

As far as valinomycin is concerned, Figs. 4.4 and 4.9 demonstrate that its presence is crucial to prevent the formation of a transmembrane potential (positive inside) which would be opposed to the entry of protons into the vesicles and would impede the formation of the proton gradient. It can be seen (figure 4.10) that the system works much better when  $[K^+]_{in} > [K^+]_{out}$ , the presence of  $K^+$  in the external medium limiting quite strongly the initial rate of quenching. The most likely explanation for this effect is that under the standard conditions used in these experiments,  $[K^+]_{in}/[K^+]_{out} = 50$ , with valinomycin present to make the ion permeant, there is a membrane potential given by the Nernst equation:

$$\Delta\psi = 2.3 RT/F \log \{[K^+]_{out}/[K^+]_{in}\} = 100 \text{ mV}$$

that is, the transmembrane  $K^+$  gradient facilitates the movement of protons by the enzyme into the inside of the vesicles, this effect decreasing when the  $K^+$  gradient is lowered by addition of external KCl. The role of the transmembrane potential of  $K^+$  on the rate and extent of ACMA quenching produced by the reconstituted ATPase was not further investigated, however, all ACMA quenching assays were performed subsequently under these conditions; therefore, although the assay could not provide absolute values of  $H^+$ -translocation in terms of activity units, it did provide relative values of this activity, thus permitting the study of the kinetic and regulatory

properties of the chromaffin granule ATPase, by assaying its physiologically relevant activity using a very straightforward procedure (see Chapter 5).

The presence of KCl throughout the purification proved to be essential to obtain optimal ATPase and  $H^+$ -translocation activities. The effect produced by KCl is probably two-fold: on one hand, it can facilitate the formation of the pellet during the fractionation with Triton X-114, and on the other, it provides osmotic support for the formation of liposomes. However, effects by other anions were not investigated. The requirement of anions for activity has been studied for the purified, reconstituted enzyme from chromaffin granules (Moriyama and Nelson, 1987a,c) and clathrin-coated vesicles (Arai *et al.*, 1989). In the first report of Moriyama and Nelson (1987a) they found that proton translocation was absolutely dependent on the presence of  $Cl^-$  and  $Br^-$  outside the vesicles, whereas  $SO_4^{2-}$ , acetate, formate,  $NO_3^-$  and thiocyanate were inhibitory. However, some criticism can be made of this work: they form vesicles with 1 mM KCl inside and then investigate the effects of the addition of 100 mM salt on the outside. This procedure has several problems, first, there is an evident osmotic imbalance between both sides of the vesicle membrane, which can produce a change in the vesicle size (i.e., a shrinking) thus altering the probe behaviour (see above); second, they did not account for the relative permeabilities of the anions through the vesicle membranes, and third, they did not take into consideration the possible buffering capacities of some permeant anions.

In their second report (Moriyama and Nelson, 1987c) they used a more sensible approach and had the same concentrations of various potassium salts (KCl,  $K_2SO_4$ , KAc) outside and inside the vesicles. Under these conditions they found that maximal  $H^+$ -translocation was observed when KCl was inside the vesicles regardless of the salt added outside. In vesicles containing  $SO_4^{2-}$  only a small decrease in activity was recorded when salts were added to the external medium except when the salt was  $K_2SO_4$  which produced a decrease in the initial rate of acridine orange quenching. Vesicles containing  $CH_3COO^- K^+$  only showed  $H^+$ -translocation activity (20% of the optimal) in the presence of external KCl. However, although they interpreted these results in terms of inhibitory anion binding sites in the enzyme, it can be argued that they overlooked the effects that different salts may have on vesicle formation, further,  $K_2SO_4$  was used in the same concentrations as the other salts, which means that when the former was present an ionic imbalance occurred. Moreover, the results of Moriyama and Nelson are based on experiments carried out with acridine orange as fluorescent probe. As has been mentioned above, this probe has been shown to aggregate in the presence of salts and it has also been reported that acridine orange in combination with  $NO_3^-$  dissipates  $H^+$  gradients across plant tonoplasts (Palmgren, 1991). All these arguments suggest that these results need further and more careful investigation.

Arai *et al.* (1989) have studied the effects of anions on ATPase activity of the purified clathrin-coated vesicle ATPase. They have shown that  $I^-$  (350 mM),  $NO_3^-$  (50 mM) and  $SO_4^{2-}$



(40 mM) inhibit ATPase activity of the detergent-solubilized enzyme. The presence of ATP lowers the concentration of  $I^-$  required for inhibition. It was reported that  $I^-$  and  $NO_3^-$  (in the presence of ATP) produced inhibition by dissociation of the complex, whereas  $SO_4^{2-}$  and  $NO_3^-$  (in the absence of ATP) do not dissociate the complex, suggesting the existence of an inhibitory anion binding site in the enzyme.

The experiments reported in this Chapter are aimed at establishing the lipid requirement for reconstitution of ATPase activity and  $H^+$ -translocation. The rate of ACMA quenching measures the development of a pH gradient ( $\Delta pH$ ), rather than  $H^+$ -translocation itself; it therefore depends on a number of factors: (1) the activity of the  $H^+$ -ATPase; (2) the proton-permeability of the vesicles; (3) the size of the vesicles. The vesicle size appears in the Schuldiner equation in the term  $\log V$ , but this term actually reflects the partition of the fluorescent probe between the vesicles and the solution, as well as the vesicle size. In intact chromaffin granules, Haigh *et al.* (1989) found the ACMA fluorescence quenching varied in a near-theoretical way with the transmembrane pH difference, but that the value of  $V$  (determined from  $\log[Q/1-Q]$  when  $\Delta pH=0$ ) was much larger than that measured by passive permeation experiments. Whatever its significance, this term is likely to be dependent on the lipid composition of the vesicles.

In general, the lipid requirements for  $H^+$ -translocation probably reflect the requirement for sealed vesicles, rather than the intrinsic lipid dependence of the ATPase. Nevertheless it was important to establish this empirical requirement in order to carry out kinetic measurements on  $H^+$ -translocation (Chapter 5). ATPase activity is much less sensitive to changes in the lipid composition of the vesicles than is the rate of measured  $H^+$ -translocation. This is reflected in its insensitivity to cholesterol, essential for  $H^+$ -translocation, and in the non-parallel increase in the two activities when the lipid to protein ratio is increased: the fact that maximal ATPase activity is achieved without measurable  $H^+$ -translocation suggests that lipids interact at once with the enzyme, the latter adopting its optimal conformation before sealed vesicles are formed.  $H^+$ -translocation is then observed at higher lipid:protein ratios with little loss of ATPase activity, which might suggest that a major fraction of the sealed vesicles are orientated the right way round.

The optimal phospholipid composition found is quite similar to that found in the membranes of chromaffin granules (Winkler and Westhead, 1980), though the latter contains lysophosphatidylcholine, not tested in our experiments. Granule membranes contain a larger fraction of cholesterol than was required for ATPase or  $H^+$ -translocation activity in our vesicles, but greater mole fractions of cholesterol were not inhibitory. These results can also be compared with those of Xie *et al.* (1986) who investigated the lipid dependence of  $H^+$ -translocation by the ATPase of clathrin-coated vesicles. They found that a much larger ratio of lipid to protein (200:1) was required for optimal  $H^+$ -translocation, but their optimal lipid composition was remarkably similar to that found in the present work. The difference may arise

in the different method of vesicle preparation rather than on the intrinsic lipid requirements of the enzymes. Furthermore, they optimized the maximal extent of accumulation of their pH probe, acridine orange, rather than its initial rate.

The purification method developed provides a rapid and convenient method for preparing vesicles containing purified chromaffin granule ATPase I active in  $H^+$ -translocation, in which fluorescent probes can be used to measure the enzyme activity. Fluorescent methods are fast and very straightforward and measure the primary function of the enzyme,  $H^+$ -translocation, as opposed to ATP hydrolysis. These features make this preparation ideal for further studies on the chromaffin granule ATPase, especially the kinetic and regulatory characteristics of this enzymic complex (see next Chapter). These have so far only been investigated by measurements of ATP hydrolysis in chromaffin granule "ghosts" or membranes, which contain another ATPase of unknown function, termed ATPase II.



## **Chapter 5**

### **KINETIC STUDIES ON THE CHROMAFFIN GRANULE $H^+$ -ATPase**

## 5.1. INTRODUCTION.

It has already been mentioned that vacuolar ATPases have been recognized as a distinct group of ATPases only recently and that our knowledge of their properties and characteristics is limited. In the last few years, the bulk of research has been focused on the purification of these complexes and on identification of the polypeptides associated with the ATPase and  $H^+$ -translocation activities. Nowadays, although there is still plenty to be done, fair agreement exists amongst the different research groups about the likely structure of these enzymes.

Kinetic studies have been carried out on vacuolar ATPases by measuring ATP hydrolysis by enzymes from *Beta vulgaris* (Bennet *et al.*, 1985), clathrin-coated vesicles (Arai *et al.*, 1989) and chromaffin granules, using either intact granules (Johnson *et al.*, 1982) or purified reconstituted enzyme (Hanada *et al.*, 1990). However, such studies can only give partial information about the kinetic properties of this type of enzyme because ATP hydrolysis is just an incidental manifestation of the physiologically relevant activity,  $H^+$ -translocation.

Flatmark *et al.* (1985) did carry out kinetic studies by assaying  $H^+$ -translocation in resealed chromaffin granule membranes ("ghosts") and calculated  $K_m$  values for this activity. However, it should be remembered that there are two ATP hydrolysis activities in the chromaffin granule membrane (ATPases I and II). This means that when  $H^+$ -translocation assays are being carried out, although only ATPase I is known to pump protons, ATPase II will hydrolyse ATP, and contribute to the measured rate. For this reason, kinetic parameters measured with intact "ghosts" are not very reliable.

In Chapter 4, I reported and discussed the development of a new purification method for ATPase I of chromaffin granules. This method yielded a stable, partially purified form of the enzyme reconstituted into liposomes of defined composition. This preparation enabled us to measure the accumulation of protons into the liposomes by the enzyme and was completely free of ATPase II. The suitability of the fluorescent probe ACMA for measuring the transmembrane proton gradient was also studied and discussed, and it was pointed out that the initial rate of fluorescence quenching in this type of experiments can be theoretically demonstrated to be proportional to the  $H^+$ -translocation activity.

It was experimentally shown that the initial rate of fluorescence quenching was proportional to the amount of protein (i.e., activity) present and that it was decreased in parallel to the ATP hydrolysis activity upon the addition of bafilomycin  $A_1$ , a specific inhibitor of vacuolar ATPases. As a consequence, the initial rate of ACMA fluorescence quenching was used as a quantitative measure of the velocity at which the enzyme translocates protons. The conclusion was that a system that overcame the problems hitherto associated with studies on kinetic and regulatory properties of vacuolar ATPases (see above) had been developed.

In the present chapter I report and discuss the study of the kinetic and regulatory properties of reconstituted chromaffin granule ATPase I, using the ACMA quenching assay for measurement of  $H^+$ -translocation. These results are compared with those obtained for chromaffin granule "ghosts". ATP hydrolysis both by the reconstituted enzyme and by "ghosts" was also studied. Regulatory and inhibitory studies were carried out by examining the effect of different nucleotides on  $H^+$ -translocation. Possible structural and physiological implications are also discussed.

## 5.2. METHODS.

Reconstituted ATPase was prepared as described in Chapter 4, except that the lipid composition utilized for reconstitution was 68.4% PE, 11.1% PS and 20.6% cholesterol. This lipid mixture, in which PC is completely substituted by PE, does not alter significantly the  $H^+$ -translocation activity of the enzyme with respect to the optimal composition reported previously (although ATP hydrolysis activity is smaller: see Chapter 4) and overcomes problems of oxidation of PC that were encountered in some batches of this lipid. Chromaffin granule "ghosts" and membranes were obtained and  $H^+$ -translocation and ATP hydrolysis assays were performed as indicated in the Materials and Methods Chapter. An excess of 1 mM  $MgSO_4$  was present in both activity assays in order to make sure that most MgATP added to the medium to start the reactions remained in its undissociated form. Considering a dissociation constant ( $K_d$ ) for MgATP of  $1.25 \times 10^{-5}M$  (O'Sullivan and Perrin, 1964) it is easily calculated that the proportion of free ATP in the medium under these conditions is negligibly small (<1.5%) in the range of MgATP concentrations used in these experiments. When the effects produced by  $Zn^{2+}$  and  $Cu^{2+}$  in  $H^+$ -translocation were studied a fixed concentration of 1 mM ATP was present in the ACMA assay medium, which contained no other salts. Reaction was started by adding different amounts of the respective metal sulphates.

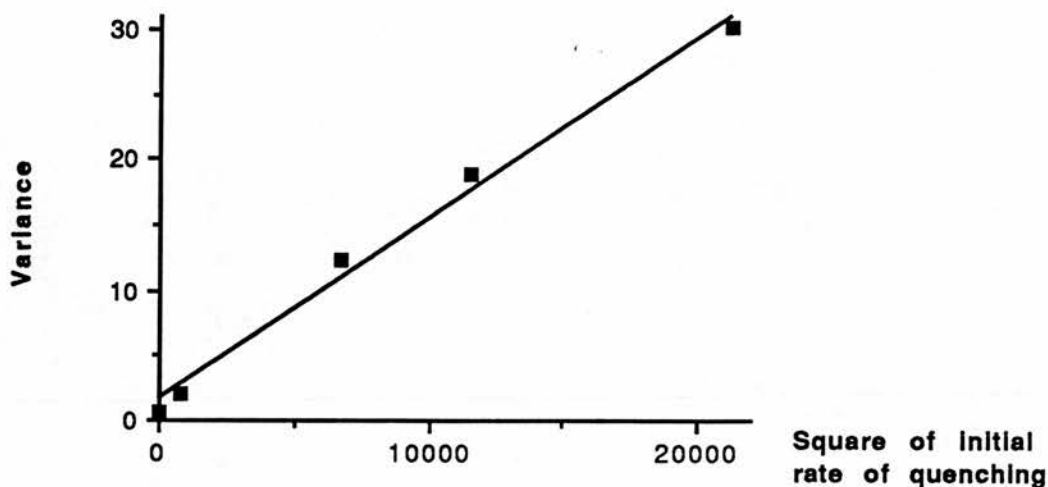
Kinetic parameters were calculated by fitting the experimental data to the appropriate function (Michaelis-Menten, Hill or allosteric: see below) by using weighted least squares regression. A computer programme written in C programming language and run on the Sequent computer of the Edinburgh University Computing Service under UNIX was utilized. The programme produced optimal values for the parameters found in the respective function as well as sets of optimized data. Graphs were then produced by plotting the experimental data points and overplotting lines calculated according to the optimized parameters.

## 5.3. RESULTS.

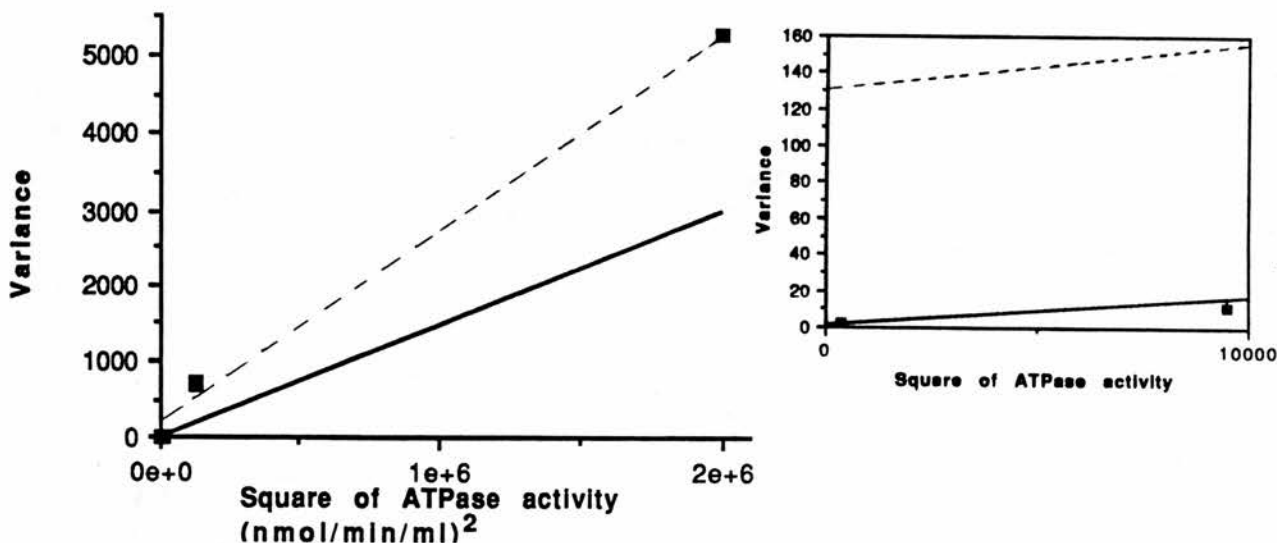
### 5.3.1. Calculation of kinetic parameters.

#### 5.3.1.1. Reproducibility of the ACMA quenching and ATP hydrolysis assays.

The error distribution in measurement of the initial rate of ACMA quenching and ATP hydrolysis was assessed by carrying out 10 replicate determinations for each of five different rates in the first case, and 8 determinations for each of four different rates in the second. Rates were produced by using the same enzyme concentration with different concentrations of MgATP.



**Figure 5.1. Proportionality between the variance and the square of initial rate of ACMA quenching.** Variances were calculated as indicated in text.



**Figure 5.2. Weighting function for the ATP hydrolysis assay.** The function was obtained by weighting the variance values themselves according to the inverse of the square of their values in order to obtain optimal fitting at low velocities. The dotted line represents least square regression of non-weighted data. Insert shows both functions at low rates of activity. See text for further details.

The data thus obtained were analysed. It was found that the variances were proportional to the square of the rates for both assays. In the case of the ACMA quenching assay the weighting function was obtained by weighting equally each of the variance values (see figure 5.1) and applying least squares regression. When this procedure was utilized to obtain the ATPase assay weighting function it was observed that the fitting of the experimental variances to the weighting function thus obtained was proportionally better at high ATP hydrolysis rates. It was decided to obtain a weighting function with better fitting at low- and medium-ATPase velocities (although the deviation at high velocities was then greater) which was more suitable for further calculations. This was achieved by weighting each variance according to the inverse of the square of its value, that is, the bigger the variance (and, thus, the ATPase rate) the smaller its weight in the calculation of the weighting function. Figures 5.1 and 5.2 show weighting functions for H<sup>+</sup>-translocation and ATP hydrolysis assays respectively.

#### 5.3.1.2. Determination of kinetic parameters for H<sup>+</sup>-translocation.

A series of experiments was carried out with reconstituted ATPase and kinetic parameters were calculated for various substrates and metal ion concentrations. A comparative representation of the variation of the initial rate of fluorescence quench with substrate concentration for MgATP, MgITP and MgGTP is shown in figure 5.3. MgCTP and MgUTP did not act as substrates for the enzyme.

The data were fitted to the empirical Hill equation:

$$v_o = \frac{V_{\max}}{1 + (K/S)^{n_h}}$$

Calculation of kinetic parameters showed that both ITP and GTP have smaller  $V_{\max}$  values and bigger K values than ATP, ITP having bigger  $V_{\max}$  and K than GTP. However, whereas  $n_h$  values for MgATP and MgITP were very similar and close to 1, in the case of MgGTP this value increases to about 1.5 which suggests a positive cooperative effect. These results are shown in Table 5.1. MgGATP had a higher K than MgATP although  $V_{\max}$  values could not be compared because the data relate to a different preparation of the enzyme (Table 5.1). However, when the same preparation was used  $V_{\max}$  for MgATP was shown to be 30% higher than for MgGATP.  $n_h$  proved to be similar to the values obtained for MgATP and MgITP.

K values for H<sup>+</sup>-translocation in reconstituted ATPase using MgATP as substrate were compared with those obtained in "ghosts" and K values were shown to be very similar although  $n_h$  for "ghosts" was much bigger (figures 5.4 (a) and (b) and Table 5.1).

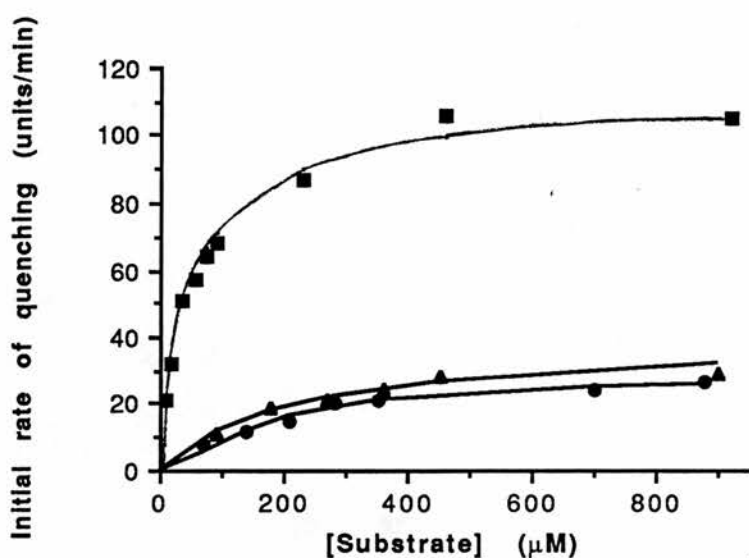


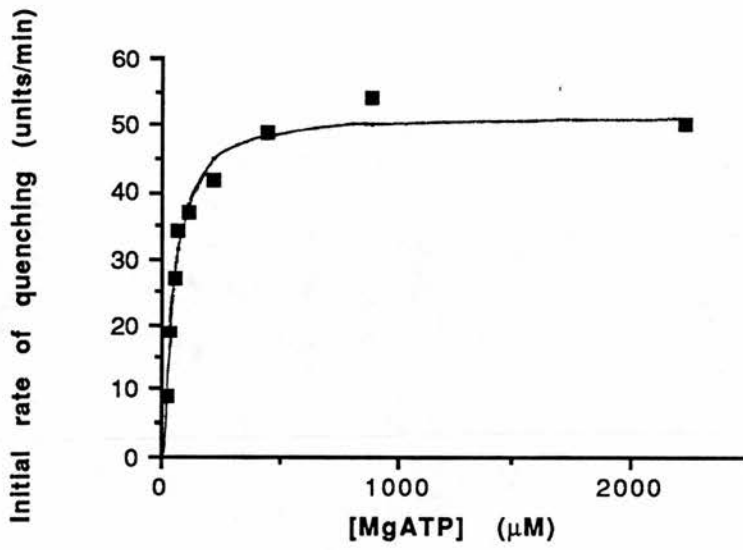
Figure 5.3. Variation of the initial rate of ACMA quenching with increasing amounts of MgATP(■), MgITP (▲) and MgGTP (●).

Substrate	K (μM)	V <sub>max</sub> (1) (units/mln)	n <sub>h</sub>
MgATP	51.4 ± 3.2	115 ± 3	0.86 ± 0.02
MgITP	268 ± 59	43.1 ± 3.9	0.88 ± 0.12
MgGTP	170 ± 8	28.0 ± 0.9	1.56 ± 0.21
MgdATP (2)	91.2 ± 6.8	102 ± 2	0.90 ± 0.03
MgATP ("ghosts")	51.4 ± 1.8	51.2 ± 0.7	1.38 ± 0.06

Table 5.1. Comparison of kinetic parameters for different substrates for H<sup>+</sup>-translocation. (1) V<sub>max</sub> values are expressed in arbitrary fluorescence units, that is, they cannot be interpreted in terms of activity units, being useful only in comparative terms. (2) Data corresponding to a different preparation, thus, V<sub>max</sub> is not directly comparable to those above. When same preparation was used V<sub>max</sub> for MgATP was found to be about 30% higher than that of MgdATP.



( a )



( b )

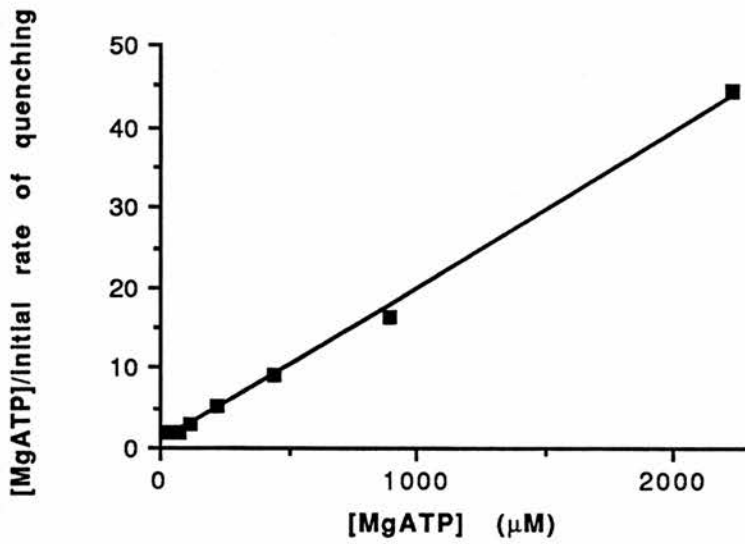


Figure 5.4. Michaelis (a) and Hanes (b) representations for  $\text{H}^+$ -translocation activity in chromaffin granule "ghosts".

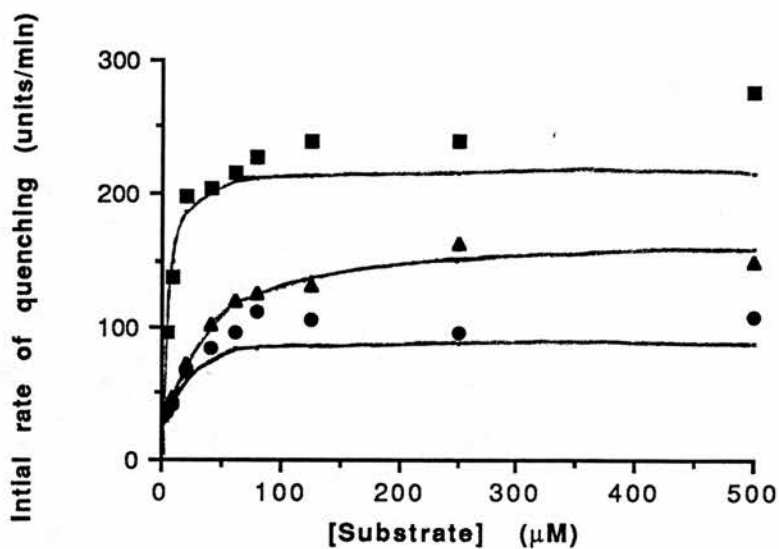


Figure 5.5. Variation of the initial rate of ACMA quenching with increasing amounts of MnATP (■), MgATP (▲) and CaATP (●).

Substrate	K ( $\mu\text{M}$ )	Vmax (units/mln)	$n_h$
MgATP	$25.6 \pm 1$	$167 \pm 2.2$	$0.98 \pm 0.03$
CaATP	$8.14 \pm 0.86$	$88.4 \pm 3.1$	$1.39 \pm 0.25$
MnATP	$5.97 \pm 0.37$	$215 \pm 5$	$1.57 \pm 0.23$

Table 5.2. Effect of different divalent cations on kinetic parameters for  $\text{H}^+$ -translocation.

Figure 5.3 shows a Michaelis plot for MgATP, MnATP and CaATP, the values of the kinetic parameters are expressed in Table 5.2. It can be seen that  $Mn^{2+}$  activates the enzyme by increasing the  $V_{max}$  and decreasing  $K$  with respect to the natural substrate, MgATP.  $Ca^{2+}$  produces a decrease in both  $V_{max}$  and  $K$ .  $n_H$  values for CaATP and MnATP are also much higher than that of MgATP. The enzyme could also utilize  $Ni^{2+}$  and  $Co^{2+}$  as co-substrates, however, these two ions interfere with the fluorescence assay, most likely because they form coloured coordination complexes in aqueous solution; thus, a reliable calculation of kinetic parameters was not possible for NiATP and CoATP. In the presence of  $Cu^{2+}$  no quenching was observed and  $Zn^{2+}$  only produced a small quenching which was independent of the amount of  $ZnSO_4$  added.

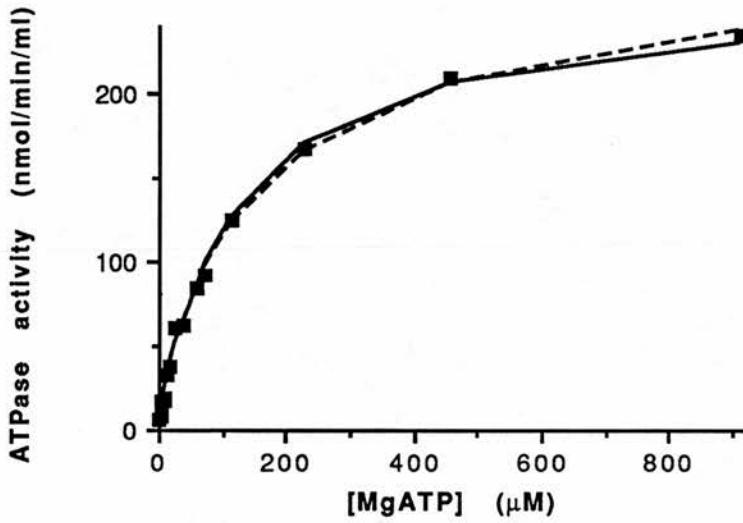
#### 5.3.1.3. Calculation of kinetic parameters for ATP hydrolysis.

The variation of the initial rate of ATP hydrolysis with substrate concentration for reconstituted ATPase and chromaffin granule "ghosts" is shown in figures 5.6 (a) and 5.7 (a). Hanes representations of both sets of data reveal a deviation from ideal Michaelis-Menten kinetics (figures 5.6 (b) and 5.7 (b)). The data could be fitted according to two functions: (1) the sum of two Michaelis-Menten functions with two values for  $K_m$  and  $V_{max}$ ; (2) A Hill function with one single value for  $V_{max}$  and  $K$  and a Hill coefficient. Kinetic parameters are shown in Table 5.3. Fitting to the sum of three Michaelis-Menten functions produced unsatisfactory results. It is notable that there is a considerable difference between the values obtained for "ghosts" using both approaches. However, the fitting to the Hill equation was not as good as to the double Michaelis function (figure 5.7), so values obtained by using the latter approach should be more reliable.

#### 5.3.2. Inhibitory and regulatory studies.

In order to study the regulatory properties of the enzyme different nucleotides were tested for inhibition of  $H^+$ -translocation. CDP, UDP and AMP did not exert any inhibitory effect on the enzyme at concentrations up to 300  $\mu M$  but ADP, IDP and GDP did. Figures 5.8 to 5.11 show Michaelis and Hanes representations of the variation of the initial rate of ACMA quenching with MgATP concentration in the presence of different concentrations of ADP, IDP and GDP respectively. It is evident that, in the presence of inhibitors, there is cooperativity in ATP binding so that Michaelis-Menten kinetics no longer apply. The Hill equation could be applied in order to calculate kinetic parameters. Tables 5.4 and 5.5 Hill show  $n_H$ ,  $V_{max}$  and  $K$  values. It can be seen that increasing inhibitor concentrations produce a systematic increase in the values of  $K$  and  $n_H$  and a decrease in  $V_{max}$  for ADP. This effect on  $V_{max}$  is not so clear for GDP and IDP.

(a)



(b)

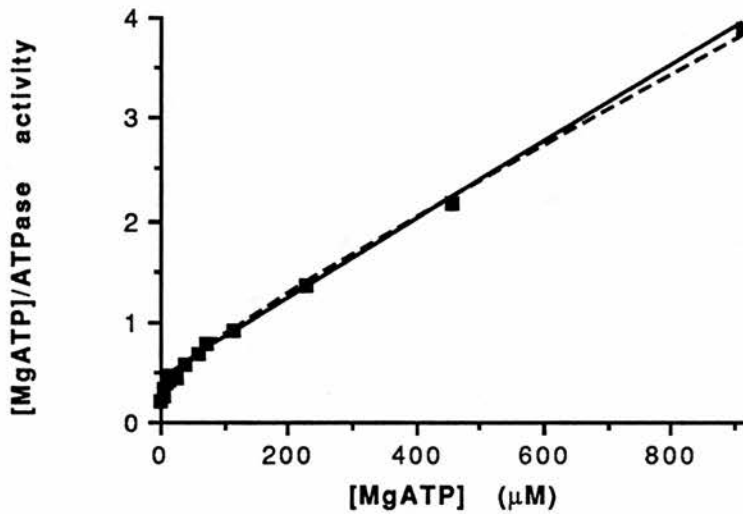


Figure 5.6. Michealis (a) and Hanes (b) representations for ATP hydrolysis in purified reconstituted ATPase. Filled squares represent experimental data points and lines were calculated according to the parameters obtained by fitting the data to the sum of two Michaelis-Menten functions (—) or to the Hill (- - -) equation.

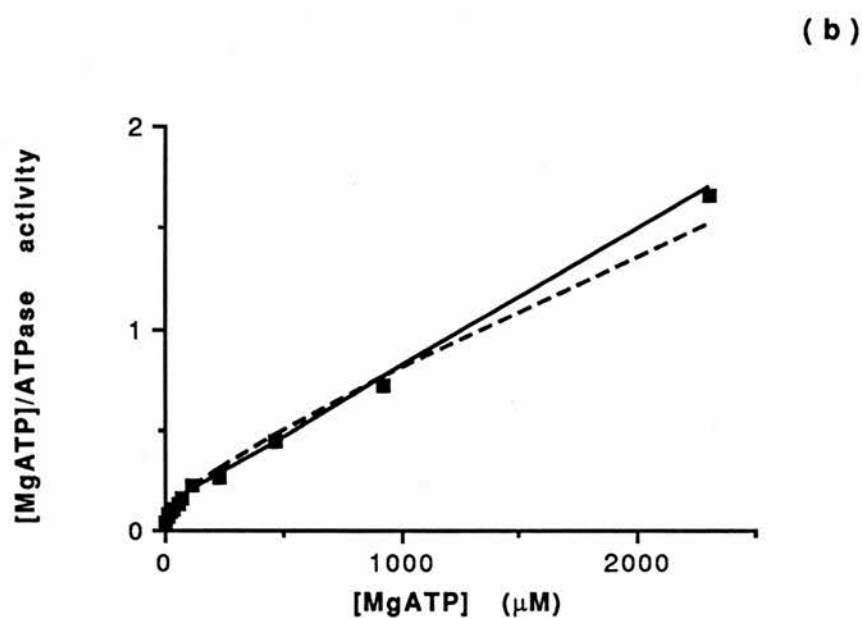
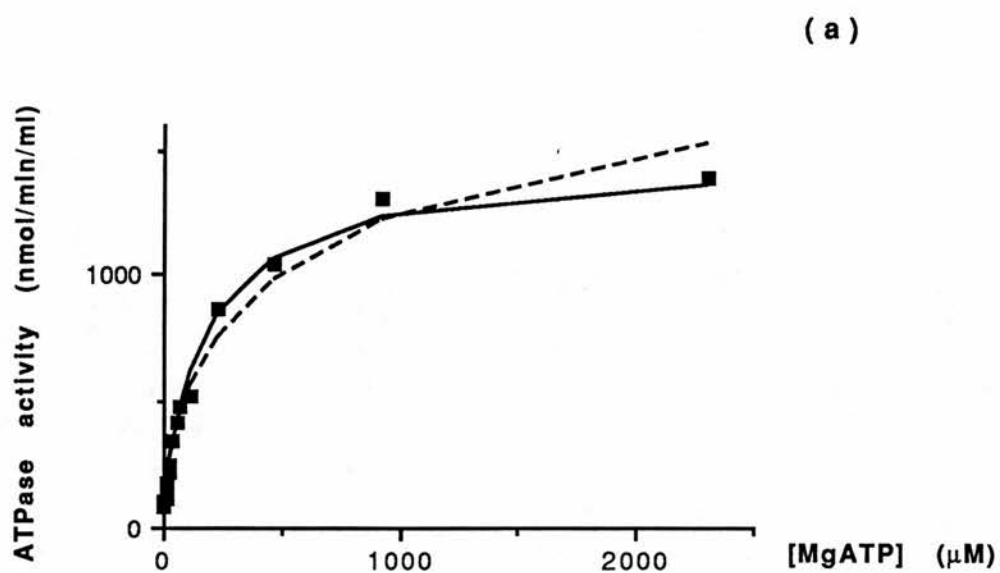


Figure 5.7. Michaelis (a) and Hanes (b) representations of ATP hydrolysis activity in chromaffin granule "ghosts". Squares and lines as in Figure 5.6.

### Hill equation

	$n_h$	$K$ ( $\mu M$ )	$V$ (nmol/min/ml)
- "Ghosts"	$0.61 \pm 0.01$	$707 \pm 124$	$2261 \pm 132$ ( <b>870</b> )
- Purified ATPase	$0.84 \pm 0.01$	$174 \pm 10.8$	$298 \pm 7.5$ ( <b>1490</b> )

### Double Michaelis

	$K_{m1}$ ( $\mu M$ )	$V_1$ (nmol/min/ml)	$K_{m2}$ ( $\mu M$ )	$V_2$ (nmol/min/ml)
- "Ghosts"	$184 \pm 7$	$1393 \pm 25$ ( <b>536</b> )	$0.20 \pm 0.17$	$73.8 \pm 4.1$ ( <b>28.4</b> )
- Purified ATPase	$128 \pm 5.6$	$255.6 \pm 4.1$ ( <b>1278</b> )	$1.4 \pm 0.7$	$6.5 \pm 1.2$ ( <b>32.5</b> )

Table 5.3. Values of kinetic parameters for ATP hydrolysis calculated by fitting experimental data to Hill and Michaelis-Menten equations. Numbers in bold between parantheses indicate specific activities in nmol/min/mg.

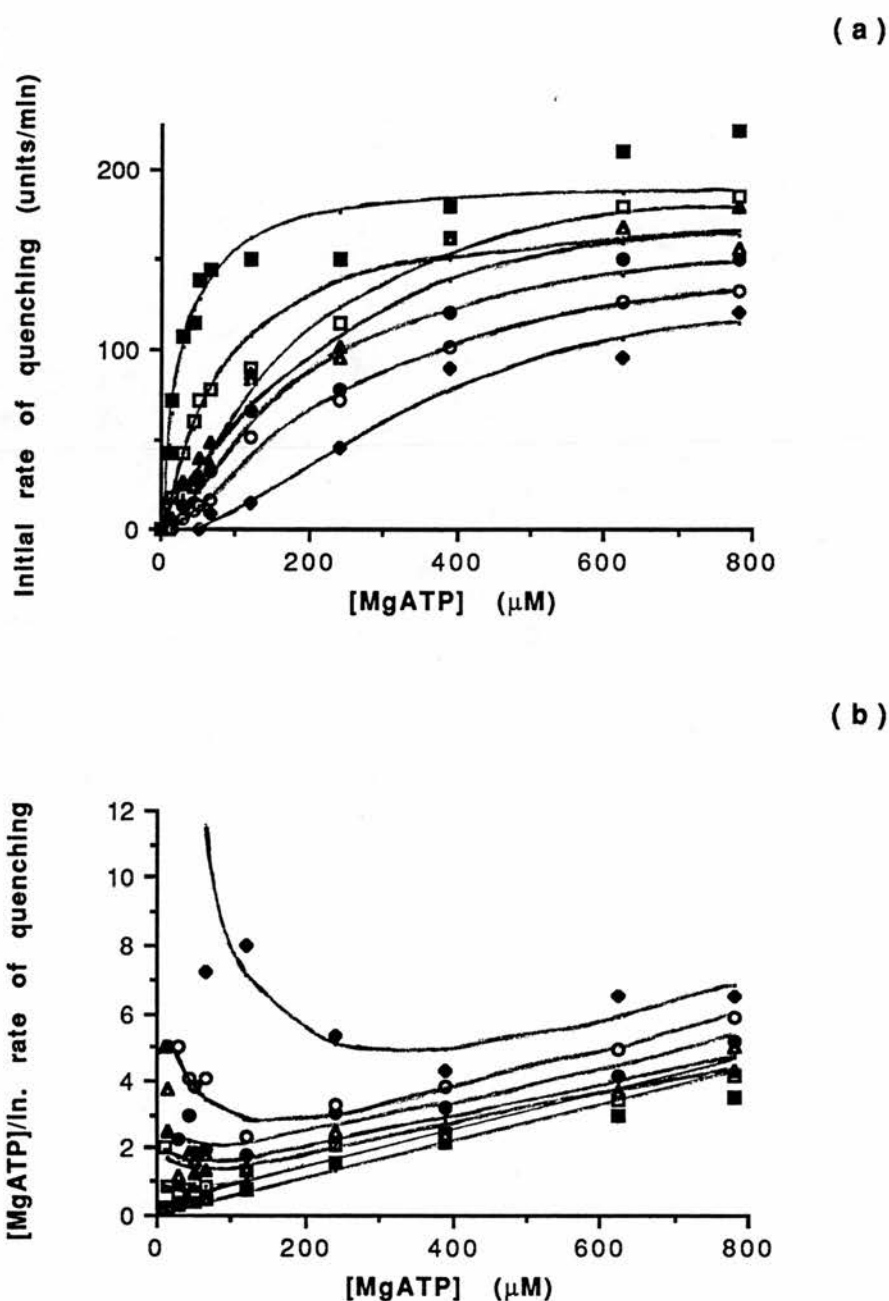
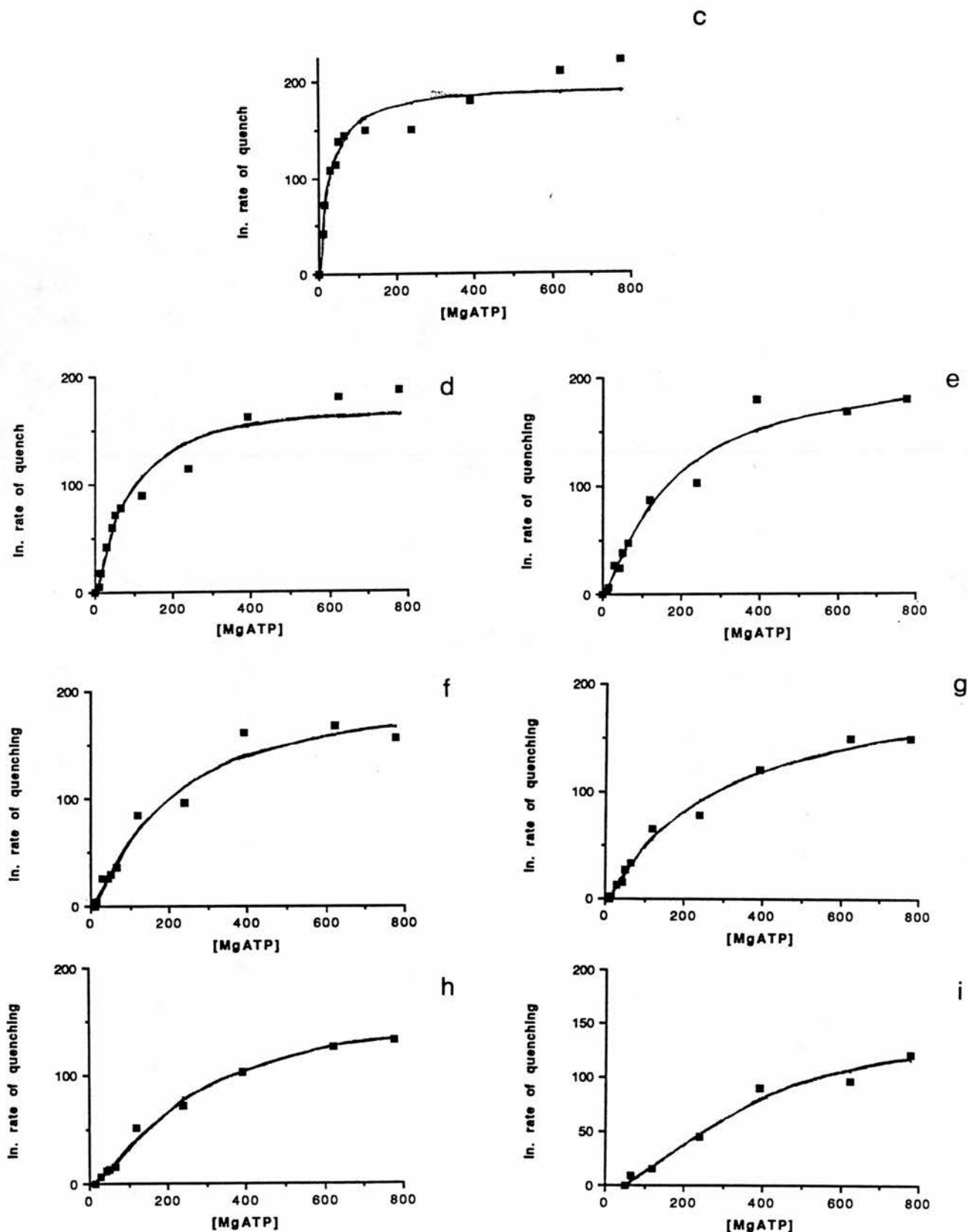


Figure 5.8. Michaelis-Menten (a) and Hanes (b) plots of the variation of  $\text{H}^+$ -translocation activity with increasing concentrations of MgATP in the presence of 0 ( $\blacksquare$ ), 17.5 ( $\square$ ), 35 ( $\blacktriangle$ ), 52 ( $\triangle$ ), 70 ( $\bullet$ ), 140 (O) and 280 ( $\blacklozenge$ )  $\mu\text{M}$  ADP.





Figures 5.8 (c) to 5.8 (i). Individual Michealis representations of variation of Initial rate of ACMA quenching with increasing amounts of MgATP in the presence of 0 (c), 17.5 (d), 35 (e), 52.5 (f), 70(g), 140 (h) and 280 (i)  $\mu\text{M}$  ADP. Full squares represent experimental data points and lines were calculated by fitting to the empirical Hill equation.

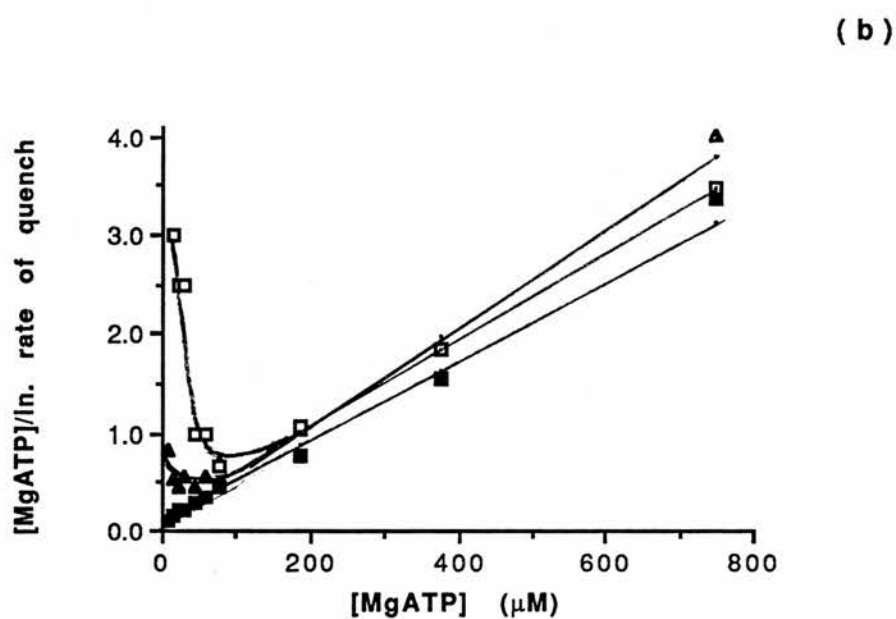
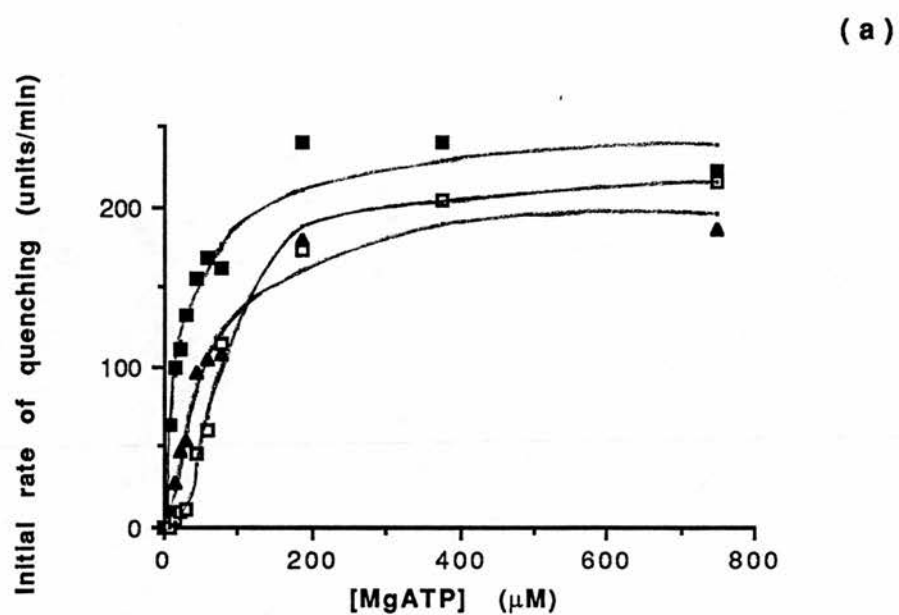
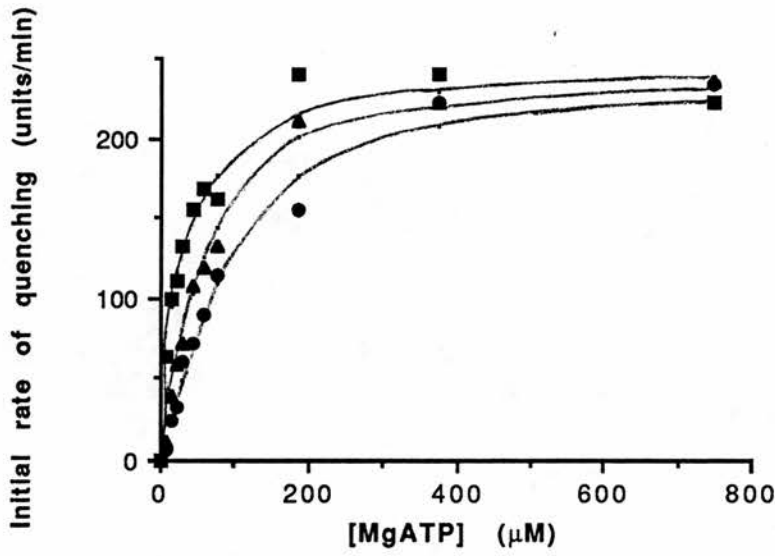


Figure 5.9. Michaelis Menten (a) and Hanes (b) plots of the variation of  $\text{H}^+$ -translocation activity with increasing amounts of MgATP in the presence of 0 ( $\blacksquare$ ), 150 ( $\blacktriangle$ ) and 300 ( $\square$ )  $\mu\text{M}$  GDP.

(a)



(b)

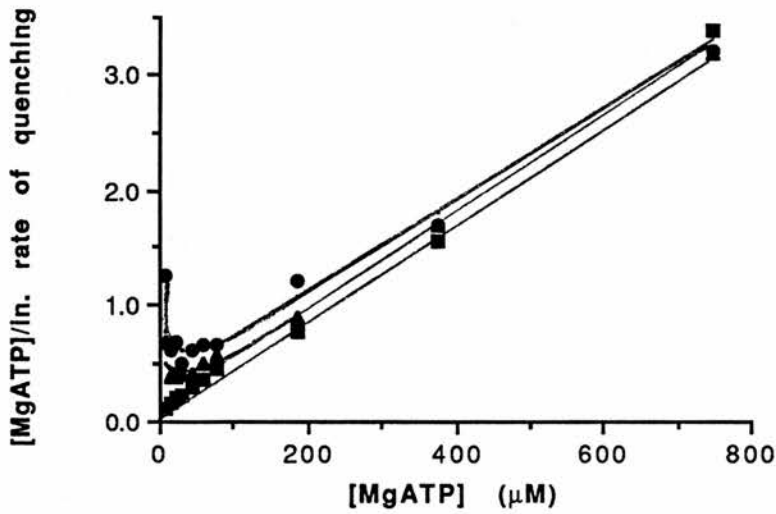


Figure 5.10. Michaelis Menten (a) and Hanes (b) plots of the variation of  $\text{H}^+$ -translocation activity with increasing amounts of MgATP in the presence of 0 ( $\blacksquare$ ), 120 ( $\blacktriangle$ ) and 240 (O)  $\mu\text{M}$  IDP.

<b>[ADP]</b> <b>(<math>\mu\text{M}</math>)</b>	<b>V</b> <b>(units/min)</b>	<b>K</b> <b>(<math>\mu\text{M}</math>)</b>	<b><math>n_h</math></b>
0	193 $\pm$ 6	27.6	1.12 $\pm$ 0.07
17.5	173 $\pm$ 8	78.2	1.26 $\pm$ 0.08
35	202 $\pm$ 13	174	1.28 $\pm$ 0.08
52.5	193 $\pm$ 14	188	1.27 $\pm$ 0.09
70	182 $\pm$ 14	240	1.25 $\pm$ 0.08
140	146 $\pm$ 7	218	1.58 $\pm$ 0.07
280	138 $\pm$ 17	336	1.91 $\pm$ 0.24

**Table 5.4. Hill parameters for ADP inhibition experiments.** Data were calculated by linear regression as indicated in the text.

<b>Inhibitor</b>	<b>V</b> <b>(units/min)</b>	<b>K</b> <b>(<math>\mu\text{M}</math>)</b>	<b><math>n_h</math></b>
None	253 $\pm$ 7	28.1	0.84 $\pm$ 0.04
IDP (120 $\mu\text{M}$ )	239 $\pm$ 5	54.7	1.35 $\pm$ 0.04
IDP (240 $\mu\text{M}$ )	237 $\pm$ 8	83.3	1.31 $\pm$ 0.05
GDP (150 $\mu\text{M}$ )	202 $\pm$ 5	56	1.41 $\pm$ 0.05
GDP (300 $\mu\text{M}$ )	206 $\pm$ 6	77.7	2.63 $\pm$ 0.13

**Table 5.5. Hill parameters for IDP and GDP inhibition experiments.** As above, parameters were calculated by linear regression.

### 5.3.3. Design of a mathematical model for the regulatory properties of the chromaffin granule ATPase.

The main objective of this research project is the functional and structural characterization of the chromaffin granule ATPase, and it was thus important to design a model that could explain quantitatively the results obtained in the regulatory studies. It was decided to approach this problem by using the allosteric model proposed by Monod *et al.* (1965). This model is relatively simple and explains cooperative interactions in terms of molecular symmetry. Its main advantage is that, solving the equations that the model proposes to predict enzyme behaviour, a structural number  $n$ , distinct from the parameter  $n_H$  in the empirical Hill equation, can be obtained. This number indicates the number of functional subunits in the enzymic complex, this information being very important for our objectives in the present project.

The allosteric model proposes that enzymes that show cooperative effects are composed of a number of identical subunits. A subunit or protomer in the allosteric model is a structure that is repeated several times within the enzymic complex and does not necessarily refer to a single polypeptide chain. In the simplest case, the enzyme can exist in two states, R and T, with different binding and kinetic properties. This model predicts the following saturation function:

$$\bar{Y}_S = \frac{(S/K_R) [1 + (S/K_R)]^{n-1} + L' (S/K_T) [1 + (S/K_T)]^{n-1}}{[1 + (S/K_R)]^n + L' [1 + (S/K_T)]^n} \quad (1)$$

where

$$L' = L (1 + I/K_I)^n$$

$S$  is the substrate concentration,  $K_R$  is the dissociation constant of the substrate from the R state,  $L$  is the allosteric constant (R-T equilibrium constant, equal to  $[T]/[R]$ ),  $I$  is the inhibitor concentration,  $K_I$  is the dissociation constant of the inhibitor from the T state,  $K_T$  is the dissociation constant of the substrate from the T state and  $n$  is the number of subunits of the complex. This expression is deduced by assuming that the binding of the inhibitor to the R state is negligible.

The allosteric model can account for two extreme cases: "K systems", in which substrate and inhibitor have different affinities for the two states and "V systems", in which the substrate has the same affinity for both states, which however differ in catalytic activity. In K systems, the presence of the inhibitor affects the apparent affinity of the substrate for the enzyme, that is, changes the apparent  $K_m$  (or  $S_{0.5}$ ). In V systems, the presence of inhibitor affects the activity of the enzyme but not the affinity for the substrate, that is, it changes  $V_{max}$  but not the apparent  $K_m$ .

The presence of inhibitors changes  $K_m$  and  $V_{max}$  for the chromaffin granule  $H^+$ -ATPase (see above), that is, the enzyme shows a mixture of the two effects predicted by the allosteric model. This may be explained by postulating that the protein has two states that differ both in their affinities for the substrate and their catalytic activities (Tipton, 1979). Assuming that the saturation function ( $\bar{Y}_S$ ) is proportional to  $v_o/V_{max}$  the expression obtained is:

$$\frac{v_o}{V_{max}} = \frac{(S/K_R) [1 + (S/K_R)]^{n-1} + L' (S/K_T) [1 + (S/K_T)]^{n-1}}{[1 + (S/K_R)]^n + L' [1 + (S/K_T)]^n} \quad (2)$$

If we further assume that R is active and T is inactive the above expression would simplify to:

$$\frac{v_o}{V_{max}} = \frac{(S/K_R) [1 + (S/K_R)]^{n-1}}{[1 + (S/K_R)]^n + L' [1 + (S/K_T)]^n} \quad (3)$$

The fact that in the absence of inhibitors the enzyme shows simpler kinetics, as far as  $H^+$ -translocation is concerned, can be further explained by assuming that, under these conditions, one of the enzyme states (R) is greatly favoured in respect to the other (T), which therefore does not exert any effect, that is, the value of L is very small in the absence of inhibitor. The result is a typical Michaelis behaviour:

$$\frac{v_o}{V_{max}} = \frac{(S/K_R) [1 + (S/K_R)]^{n-1}}{[1 + (S/K_R)]^n} = \frac{S/K_R}{1 + S/K_R} = \frac{S}{K_R + S} \quad (4)$$

It is important to emphasize that, in order to explain the cooperative effects observed at even quite low concentrations of ADP, we have to assume that the dissociation constant of ADP from the T state is small (that is, the affinity of ADP for the T state is quite big) thus increasing the apparent L value ( $L'$ ) and displacing the allosteric equilibrium towards the T (inactive) state.

From the discussion above we would predict that:  $K_R$  is similar to the apparent K values obtained for MgATP in the absence of inhibitors, fitting the Hill equation (around 30  $\mu M$ );  $K_T$  is much bigger than  $K_R$  so that substrate binding cannot displace the allosteric equilibrium towards the T state; L is very small;  $K_I$  for ADP is also very small;  $V_{max}$  must be consistent with the experimental values and n is probably 2 or 3 according to the expected structure of the enzymic complex.

Experimental data were fitted to expression (3). Initial calculations were made allowing all parameters to "float" and it was then observed that values calculated for  $n$  were consistently near 2 or 3; it was then decided to fix  $n$  at 3 and 2 respectively, and determine optimized values of the rest of the parameters. Values obtained are expressed in Table 5.6. The programme also supplied sets of optimized theoretical data. No minimum in residual error was obtained with  $n=2$ . The main observations when  $n=3$  were (Table 5.6, line 1): (1)  $V_{\max}$  is underestimated; (2) the value of  $L$  is about 1.7, meaning that the T state was more abundant than the R state in the absence of inhibitors and effectors; (3)  $K_I$  was larger than predicted.

However, in equation (3) it has been assumed that the number of binding sites for substrate and inhibitors is the same, that is, 2 or 3. Therefore, in order to account for a different number of binding sites for substrates and inhibitors, a new parameter ( $n'$ ), not necessarily equal to  $n$ , was introduced in the expression of the apparent allosteric constant:

$$L' = L (1 + (I/K_I))^{n'} \quad (5)$$

where  $n'$  is the number of inhibitor binding sites. The experimental data were fitted to the new expression using two approaches: (1) fixing different values of  $n$  and  $n'$  and allowing the rest of the parameters to float and (2) fixing  $n'$  and allowing the rest of the parameters, including  $n$ , to float. Table 5.6 shows the results obtained. Optimal fitting was obtained when  $n'$  was fixed at 1; however, the values obtained for some of the other parameters were difficult to accept, thus  $n$  was not near an integral value (1.5),  $L$  was large and  $V_{\max}$  was underestimated. For  $n=2$  (i.e., 2 substrate binding sites) and  $n'=1$  (i.e., 1 inhibitor binding site) the fitting was also very good (though slightly worse than the previous case) and values of  $V_{\max}$ ,  $L$  and  $K_I$  consistent with the assumptions made in the model; however, fitting for  $n=3$  and  $n'=1$  was only marginally worse than the latter case and the calculated values of parameters were also consistent with the assumptions of the model. When  $n=3$  and  $n'=2$  and  $n'$  was fixed at 2, the fitting was worse than in the previous cases and  $V_{\max}$  was again underestimated (see Table 5.6).

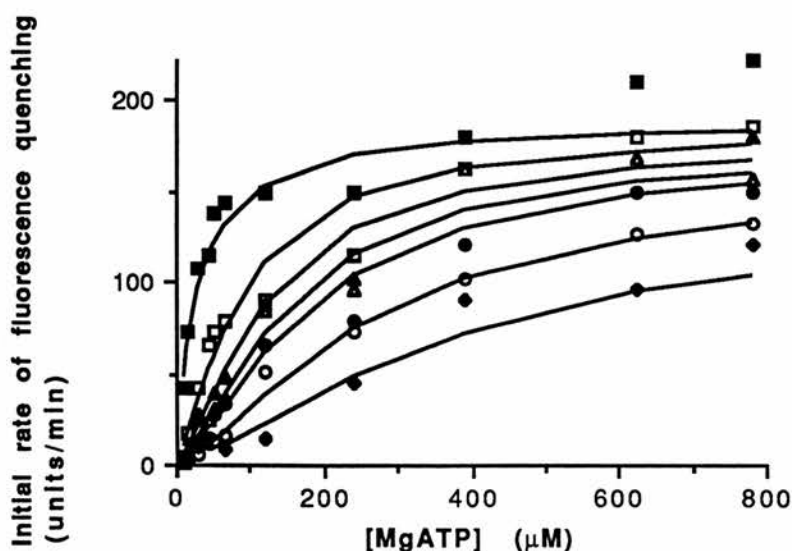
The main problem that appears in the most acceptable fittings (that is,  $n=3$ ,  $n'=1$  and  $n=2$ ,  $n'=1$ ) is the extremely small values obtained for  $L$  and  $K_I$ ; furthermore, for  $K_I$  the estimated standard error is bigger than the value of the parameter. However, as observed in figure 5.11 the fitting to the experimental values is good suggesting that the model explains the enzyme behaviour fairly well.



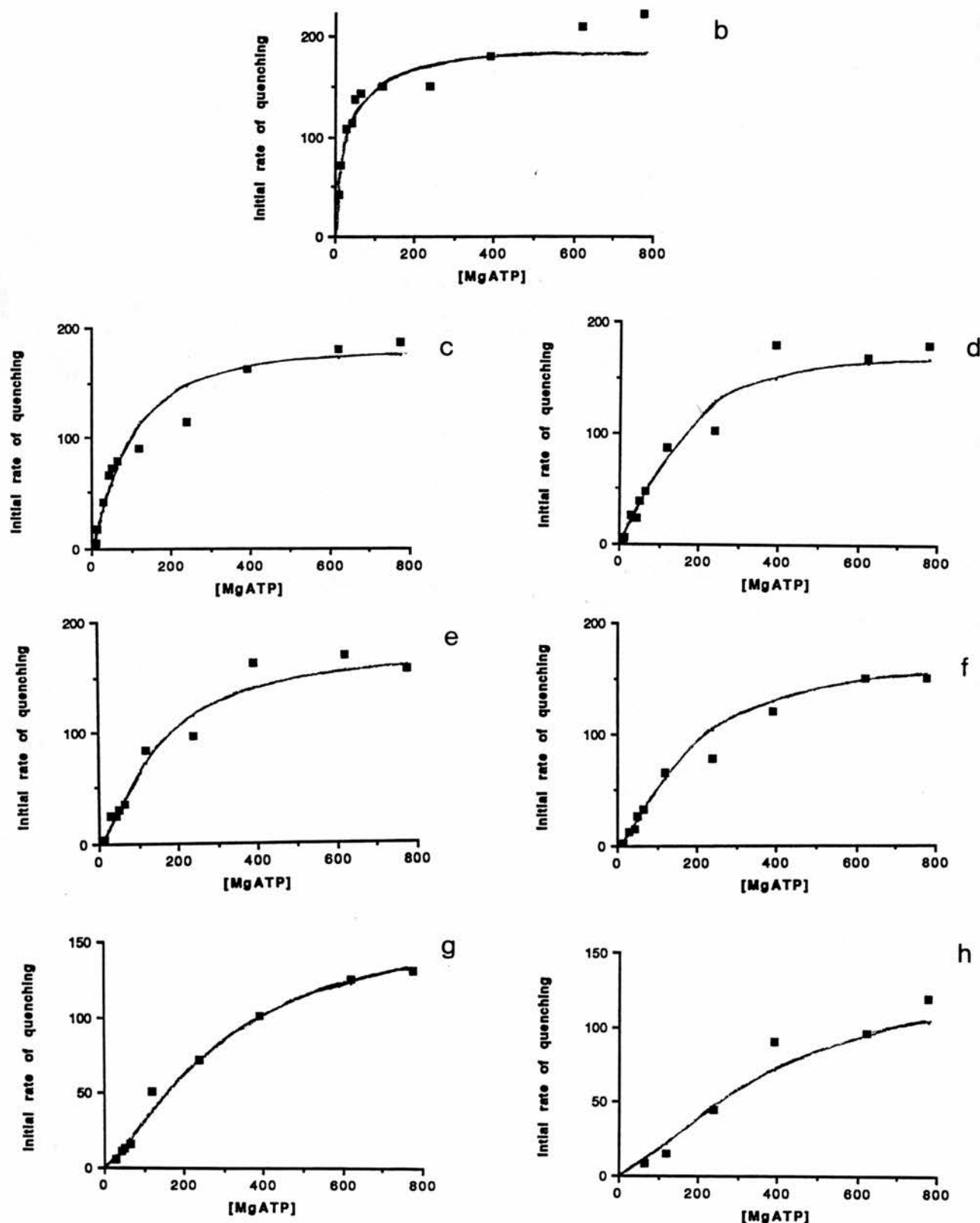
	$K_R$ ( $\mu M$ )	$V_{max}$ (units/min)	$L$	$K_T$ ( $\mu M$ )	$K_I$ ( $\mu M$ )	Sum of squares of residuals
$n = n'=3$	$28 \pm 1$	$151 \pm 1$	$1.7 \pm 0.2$	$2284 \pm 346$	$27 \pm 1$	$1.0 \times 10^4$
$n = n'=2$			(no convergence)			
$n=3, n'=2$	$22 \pm 1$	$155 \pm 1$	$0.8 \pm 0.2$	$673 \pm 31$	$6 \pm 1$	$6.9 \times 10^3$
$n'=2$ (5)	$13 \pm 2$	$154 \pm 1$	$3.0 \pm 0.7$	$7419 \pm 6574$	$11 \pm 1$	$6.5 \times 10^3$
$n=3, n'=1$	$33 \pm 1$	$195 \pm 1$	$8 \times 10^{-8}$ (1)	$205 \pm 33$	$2 \times 10^{-7}$ (3)	$3.7 \times 10^3$
$n=2, n'=1$	$29 \pm 1$	$191 \pm 1$	$6 \times 10^{-5}$ (2)	$581 \pm 20$	$2 \times 10^{-4}$ (4)	$3.2 \times 10^3$
$n'=1$ (6)	$11 \pm 2$	$182 \pm 1$	$2.3 \pm 0.7$	$3417 \pm 1048$	$2.3 \pm 0.3$	$3.0 \times 10^3$

**Table 5.6.** Values of different parameters calculated by fitting experimental data to the equation of the allosteric model. See text for details.

Standard errors:  $1.01 \times 10^{-31}$  (1),  $2 \times 10^{-4}$  (2),  $6.13 \times 10^{-8}$  (3) and  $2.0 \times 10^{-4}$  (4). (5), (6)  $n$  allowed to "float". Calculated values of  $n$ : 2.13 (5) and 1.49 (6).



**Figure 5.11(a).** Fitting of experimental data points to the allosteric equation in the presence of 0 (■), 17.5 (□), 35 (▲), 52 (△), 70 (●), 140 (○) and 280 (◆)  $\mu M$  ADP. This figure can be seen in detail in figures 5.11(b) to 5.11(h).



Figures 5.11(b) to 5.11(h). Individual representations of variation of Initial rate of ACMA quenching with increasing amounts of MgATP in the presence of 0 (b), 17.5 (c), 35 (d), 52.5 (e), 70(f), 140 (g) and 280 (h)  $\mu\text{M}$  ADP. Filled squares represent experimental data points and lines were calculated from the equations of the allosteric model (see text for details)

#### 5.4. DISCUSSION.

The measurement of the initial rate of ACMA fluorescence quenching proved to be a rapid and convenient method by which to study the kinetic and regulatory properties of the chromaffin granule ATPase. As expected, only purine nucleoside triphosphates (ATP, GTP and ITP) are substrates for the enzyme and; regarding the effect of different divalent cations,  $Mn^{2+}$ ,  $Ca^{2+}$ ,  $Ni^{2+}$  and  $Co^{2+}$  could act as co-substrates substituting for  $Mg^{2+}$ , but  $Zn^{2+}$  and  $Cu^{2+}$  could not. Indeed,  $Mn^{2+}$  was shown to produce a higher activity than did  $Mg^{2+}$ .

As far as the  $n_H$  values are concerned, it is notable that  $n_H$  values are close to 1 for MgATP and MgITP, that is, the enzyme shows a near-Michaelis-Menten kinetics in the presence of these substrates (see below), both  $Ca^{2+}$  and  $Mn^{2+}$  produce an apparent increase in this parameter; MgGTP also produces an increase in the value of  $n_H$ . It is important to note that the  $n_H$  value for "ghosts" in the presence of MgATP as substrate is also bigger than one. These effects will be discussed in detail below.  $K$  values calculated for MgATP (around 30  $\mu M$ , although values around 50  $\mu M$  were obtained in some preparations, see Tables 5.1, 5.2, 5.4 and 5.5) are consistent with those calculated previously (Flatmark *et al*, 1985) and, as expected, MgITP and MgGTP were less suitable substrates for the enzyme than the former.

As far as ATP hydrolysis is concerned it is important to underline the finding of two values of  $K_m$  with two corresponding values of  $V_{max}$  both in purified ATPase and in "ghosts". The latter fact is consistent with the obtention of a  $n_H$  value below 1 when the experimental data were fitted to the Hill equation. However, in the case of "ghosts" the values of  $K$  and  $V_{max}$  obtained by using the Hill approach are very different to those obtained by the Michaelis approach, moreover, the value of  $n_H$  in the former further deviates from 1. These effects could be due to the presence of ATPase II in the membranes which may cause inconsistencies in the calculations of kinetic parameters. Another major point to note is that fitting of the experimental values to the sum of three Michaelis-Menten was unsuccessful, that is, our experiments do not support the existence of three different  $K_m$  for ATP hydrolysis. The possibilities of an artefact of the activity assay and/or of the presence of another ATP hydrolysis activity (by contamination or due to a catalytically heterogeneous preparation of the  $H^+$ -ATPase) cannot be ruled out, however, it is important to note that similar results have been obtained with enzymes from other sources utilizing different purification procedures and different activity assays. Hanada *et al.*, (1989) reported three values of  $K_m$  and  $V_{max}$  for the chromaffin granule enzyme and Arai *et al.*, (1989) found only two in clathrin-coated vesicle ATPase, although in the latter case MgATP concentrations below 25  $\mu M$  were not tested. However, strong criticisms can be made of the work

of Hanada *et al.* because their study was not exhaustive enough, the number of data points obtained being extremely small, and the fitting to double Michaelis-Menten kinetics apparently made by Lineweaver-Burk and plots fitted by eye.

Several values for  $K_m$  and  $V_{max}$  for ATP hydrolysis have also been reported for F-type ATPases (see Table 5.7). As can be seen, all authors have reported that in both vacuolar and F-type ATPases, the increase in MgATP concentration produces a decrease in the apparent  $K_m$  observed and an increase in the rate of hydrolysis, that is, the binding of substrate seems to decrease the binding of subsequent substrate molecules to the enzyme and increases the catalytic rate. This means that there is a negative cooperativity as far as substrate binding is concerned and a positive as far as catalytic activity is concerned. This idea has been suggested for F-type ATPases where substrate-binding as well as catalytic studies have been carried out (Gresser *et al.*, 1982; Grubmeyer *et al.*, 1982; Cross *et al.*, 1982; Roveri and Calcaterra, 1985).

There is a great controversy about the development of models that can explain the occurrence of different  $K_m$  and  $V_{max}$  values for ATP hydrolysis in F-type ATPases. Most authors explain these effects by postulating the existence of three nucleotides binding sites (Gresser *et al.*, 1982; Roveri and Calcaterra, 1985), however, recently Berden *et al.* (1991), have criticized this model and proposed another based in the existence of only two catalytic sites.

The experiments carried out in the present project regarding ATP hydrolysis by the chromaffin granule ATPase do not allow us to draw clear conclusions about the structure of the protein, mainly because we have concentrated most of the work on  $H^+$ -translocation (see below). In any case, the proposition of structural models that can explain the effects observed, even when ATP hydrolysis and substrate-binding studies are carefully performed, is still controversial as has been mentioned above.

The most striking results were, however, those regarding the regulatory properties of the enzyme. Although a near-Michaelis-Menten dependence was observed for MgATP in absence of inhibitors, in the presence of ADP or other nucleoside diphosphates, binding of ATP appeared strongly cooperative. Calculation of Hill parameters showed that an increase in inhibitor concentration produced a corresponding increase in  $n_H$  and  $K$  and a decrease in  $V_{max}$ , especially in the case of ADP, which proved to be the strongest effector. The value of  $n_H$  gives an idea of the deviation from Michaelis-Menten kinetics, values below 1 suggesting negative cooperativity and values above 1 suggesting positive cooperativity. Therefore, in the case of the chromaffin granule ATPase, the presence of inhibitors induces positive cooperativity.

A theoretical model was developed in order to explain the effects produced by the inhibitors, especially ADP. The allosteric model of Monod *et al.* (1965) was chosen for this purpose because is relatively simple and gives structural information about the enzyme ( $n$  is the number of catalytic subunits). The fitting of the experimental data to the mathematical expressions derived from the model was then attempted by using different approaches.

Source (type)	Km ( $\mu$ M)			Vmax (units/mg)			Reference
Beef heart mitochondria (F)	1.7		250	2.2		55	(a)
	$10^{-6}$	30	150	$10^{-4}$	300	600 (*)	(b),(c)
	4	40	500	2	6	42	(d)
Clathrin-coated vesicle (V)	83		790	2.1		5.8	(e)
Chromaffin granules							
- "ghosts"	0.2		184	0.03		0.535	(f)
- Purified enzyme	1.4		174	0.032		1.3	(f)
	5	30	300	0.47	1.2	2.6	(g)

**Table 5.7. Comparison between kinetic parameters obtained for ATP hydrolysis activity of ATPases from different sources.** References: (a) Gresser *et al.* (1982); (b) Grubmeyer *et al.* (1982); (c) Cross *et al.* (1982); (d) Roveri and Calcaterra (1985); (e) Arai *et al.* (1989); (f) present thesis; (g) Hanada *et al.* (1990).

(\*) Data in  $s^{-1}$

A very important result as far as the structure of the enzyme is concerned is the poor (or indeed the impossibility of) fitting the experimental data to the model when the same number of binding sites for substrate and inhibitor were assumed to exist within the enzyme. Moreover, this model predicted a smaller  $V_{\max}$  and a higher value of  $L$  than appeared likely. A new parameter ( $n'$ ), distinct from  $n$ , was then introduced in order to account for the number of inhibitor binding sites. When  $n'$  was fixed at 1 a very good fit was obtained; however, the calculated value of  $n$  turned out to be 1.5, that is, far from an integral value; moreover, the high value of  $L$  obtained (2.32) was not consistent with the assumptions made to develop the model.

The most reasonable result was obtained when the number of inhibitor binding sites was assumed to be 1 and the number of substrate binding sites 2, the fitting was only slightly worse than the previous case and values of parameters were more in accordance with the assumptions of the model; however, this result was only marginally better than that obtained for three binding sites for the substrate and one for the inhibitor. Furthermore, the values of the parameters  $L$  and  $K_I$  in the former case had standard errors larger than the values themselves. Indeed, it seems that values of  $L$  and  $K_I$  in the latter case are too small to be considered reasonable, although it has been reported that an ATP-binding site in F-type ATPases has a  $K_m$  of  $10^{-6}$   $\mu\text{M}$  (Grubmeyer *et al.*, 1982, see table 5.7).

In any case, it seems that the "concerted" allosteric model explains reasonably well the kinetic properties of the chromaffin granule  $\text{H}^+$ -ATPase. The model predicts the existence of a single high-affinity inhibitor binding site and several (2 or 3) substrate binding sites. The enzyme would exist in two basic states, one catalytically active (R) and another inactive (T). In the R state the inhibitor binding site is not available and the substrate binding sites have high affinity for the substrate; in the T state the high-affinity inhibitor binding site is available and the substrate binding site decrease their affinity for the substrate. In the absence of inhibitor the occurrence of the R state is greatly favoured with respect to the T state, that is, the allosteric constant is very small, this accounts for the near-Michaelis-Menten kinetics observed under these circumstances. Because  $L$  and  $K_I$  occur together in the expression for  $L'$  (equation 5) it is not possible to obtain independent estimates of these two parameters simply from initial rate measurements (see below for further discussion).

The conclusions drawn from the model have to be considered very carefully and important considerations have to be taken into account. First of all, the "concerted" allosteric model does not account for cooperative (or negatively cooperative) interactions within each of the possible states, but explains these effects in terms of identical subunits that exist in two (or more) states in equilibrium, the substrates or effectors exerting their effects by displacing this equilibrium towards the state to which they bind with higher affinity. Therefore, this model does not account for the existence of binding sites with different affinities for substrates or effectors within a given state. ATP hydrolysis data do suggest negative cooperative interactions amongst the



catalytic sites of the enzyme. Although the values of  $n_H$  obtained for  $H^+$ -translocation did not differ much from those obtained for ATP hydrolysis, Hanes representations of these data did not confirm clearly the existence of negative cooperativity. However, it has to be taken into consideration that the investigation of  $H^+$ -translocation at values of ATP concentration between 1-10  $\mu M$  (concentrations at which cooperative effects are most notable when ATP hydrolysis was studied) was not carried out because at very low ATP concentrations both the initial rate and the extent of fluorescence quenching were very small, making it very difficult the measurement of the former; furthermore, it is likely that at very small velocities the rate of accumulation of protons may be greatly affected by effects of proton leakage from the vesicles.

The theoretical model could also have been improved by considering some activity for the T state and more possible sub-states for the enzyme within the R state, thus accounting for interactions between subunits in the active form of the enzyme. Both considerations would have meant the inclusion of new parameters and further complication of the mathematical equations. This was not considered justified on the basis of the present experimental data.

In any case, the aim was designing a relatively simple model and, although the proposed model oversimplifies the interactions that occur within the enzymic complex, it allowed us to draw important conclusions concerning the structural and functional characteristics of the chromaffin granule ATPase (see above).

There are also some comments to make about the behaviour of the enzyme in the presence of substrates other than MgATP, thus, as has been mentioned above, MgGTP, MnATP and CaATP on their own have values of  $n_H$  that are greater than 1, that is, these substrates seem to show cooperative effects. This also seems to be the case in "ghosts" when MgATP is used as substrate. In "ghosts" kinetic studies may be complicated by the presence of ATPase II and it is likely that inconsistencies may occur when studying kinetic properties of the enzyme incorporated in the native membrane. However, all these data were obtained in a limited number of experiments, and their significance must not be exaggerated.

There is a further important reservation: all kinetic models are postulated in terms of substrate- and inhibitor-binding, that is, the Hill expression and all the equations from the allosteric model give values of saturation functions ( $\bar{Y}_S$ ) for proteins (with or without catalytic activity). The catalytic rate has been assumed to be proportional to this function, i.e.,  $v_0 = k_{cat}Y_S$ . The experiments performed in this project give information on initial velocities of reaction and the value of  $v_0/V_{max}$  is strictly proportional to the saturation function only if a Michaelis equilibrium applies, that is, if the substrate-binding equilibrium is not affected by the formation of products. This means that the data obtained give only partial, although useful information about the binding properties of the chromaffin granule  $H^+$ -ATPase.

In order to draw definite conclusions about functional properties of vacuolar ATPases as well as their physiological and structural implications, proper binding studies must be carried



out, as has already been done for F-type ATPases (Grubmeyer *et al.*, 1982; Cross *et al.*, 1982). In particular, it should not be difficult to estimate binding constants of nucleoside diphosphates by equilibrium dialysis or a similar technique. Independent estimation of binding constants for MgATP and of the value of L is much more difficult.

However, despite the limitations on the information obtained we can conclude that the regulatory properties of the chromaffin granule ATPase support the idea that vacuolar ATPases are very complex proteins that probably have several substrate and inhibitor binding sites and a high internal symmetry. More detailed studies concerning the structure of this enzymic complex will be presented and discussed in the following chapter.

## **Chapter 6**

### **STRUCTURAL STUDIES ON THE CHROMAFFIN GRANULE $H^+$ -ATPase**

## 6.1. INTRODUCTION.

Vacuolar ATPases are accepted to be closely related to F-type ATPases. This close relationship is supported by immunological and genetic evidence. Thus, antibodies raised against the 72 and 57 kDa subunits from vacuolar ATPases have been shown to cross-react with the  $\alpha$  and  $\beta$  subunits from bacterial F-type ATPase (Manolson *et al.*, 1989). Furthermore, a sequence homology of around 30% has been reported to exist between these two pairs of polypeptides (Bowman *et al.*, 1988*a,b*; Zimniak *et al.*, 1988). Mandel *et al.* (1988) cloned and sequenced the 16 kDa subunit from the chromaffin granule ATPase and showed that it was closely related to the proton channel of F-type ATPases. All this evidence produced a theory of the evolution of V-type and F-type ATPases from a common ancestor (Cross and Taiz, 1989); see Chapter 1 for further discussion of this topic.

The evidence just cited suggests that both V-type and F-type ATPases may have a very similar structure, that is, vacuolar ATPases would comprise an extramembrane domain, formed by the 72 and 57 kDa subunits (catalytic and regulatory) along with a "stalk-like" structure that would couple the catalytic segment to a membrane-embedded domain, formed mainly by the 16 kDa subunit, which would be the proton channel through the membrane. This suggestion has been supported by biochemical evidence, thus, Arai *et al.* (1988) carried out topography studies on the clathrin-coated vesicles using membrane-impermeant and hydrophobic reagents. The results of these experiments indicated that the principal polypeptides labelled from the cytoplasmic surface are those of molecular masses 73 and 58 kDa. Impermeant reagents labelled the 17 kDa subunit most heavily with significant labelling of the 100 and 40 kDa subunits. In subsequent studies carried out by the same group (Adachi *et al.*, 1990*a*) treatment of the reconstituted  $H^+$ -ATPase with trypsin resulted in a cleavage of all subunits except the 19 and 17 kDa subunits, consistent with these latter being buried in the bilayer and the former having portions exposed to the cytoplasmic surface. Cross-linking studies (Adachi *et al.*, 1990*b*) suggested the proximity of the 73 and 58 kDa subunits on one hand and 17, 34, 33 and 40 kDa on the other. Arai *et al.* (1988) also attempted to determine stoichiometry by quantitative aminoacid analysis. Their results suggested that three copies of each of the 73 and 58 kDa subunits are present in the enzymic complex along with six copies of the 17 kDa subunit and one copy of the 100, 40, 38, 34, 33 and 19 kDa subunits.

In chromaffin granules, incubation of the reconstituted ATPase on ice in the presence of chloride and MgATP resulted in inactivation of the enzyme and release of the 72, 57 and 33 kDa subunits suggesting that these subunits form the cytoplasmic domain. Similar results were obtained with several other enzymes from different sources (Moriyama and Nelson, 1989*a,b,c*; Bowman *et al.*, 1989).

Electron microscopy of chromaffin granules (Schmidt *et al.*, 1982) and plant tonoplasts (Taiz and Taiz, 1991) revealed stalked particles of about 9 nm of diameter that were thought to be "heads" of H<sup>+</sup>-ATPase molecules.

However, many important aspects remain to be solved, thus, there is still some controversy about the subunit composition of vacuolar ATPases, the role of the largest (100-120 kDa) subunit is still unknown (a problem further complicated by the fact that this subunit does not appear in all preparations of this type of enzymes and that it has no counterpart in F-type ATPases) and the nature of the coupling between ATP hydrolysis and proton translocation remains obscure. This all means that, although much work has been carried out on vacuolar ATPases and our understanding of these enzymes has been greatly extended, a lot still remains to be done in order to understand the structure-function relationship in multi-subunit complexes.

In the previous chapter, the kinetic and regulatory properties of the chromaffin granule H<sup>+</sup>-ATPase were studied using a partially-purified, reconstituted form of the enzyme and a quantitative assay for its H<sup>+</sup>-translocation activity. The complexity of the mechanisms by which the enzymic activities were controlled was found to be consistent with the protein having a multimeric structure most likely involving the presence of several substrate- and inhibitor-binding sites.

In this Chapter, I report experiments involving a variety of techniques and approaches, which have been grouped in the same chapter because they are all aimed at the elucidation of the structure of the chromaffin granule H<sup>+</sup>-ATPase. First of all, enzymic degradation of oligosaccharides as well as specific labelling of glycoproteins was carried out in order to investigate the occurrence of glycosylated subunits, for glycosylation may have important implications for the arrangement of polypeptides in the chromaffin-granule membranes. Then, the hydrophobic probe 3-(trifluoromethyl)-3-(*m*-[<sup>125</sup>I]iodophenyl)diazirine (TID) was utilized to label the membrane-embedded polypeptides of the ATPase. Finally, the stoichiometry of the enzymic complex was studied by quantitative aminoacid analysis and scanning of silver-stained SDS-PAGE gels. In order to perform the latter experiments a more highly purified form of the protein was obtained because analysis carried out on reconstituted ATPase produced inconsistent results. Further purification was achieved by ultracentrifugation in glycerol density gradients and reconstitution of the purified complex was performed by dialysis. Density gradient analysis revealed the presence of additional subunits associated with the enzyme activities.

The results shown on this Chapter along with others obtained in our laboratory have enabled us to obtain useful information about the topography of the chromaffin granule ATPase, which suggests that it is a multi-subunit enzyme of high molecular weight whose structure resembles that of F-type ATPases. The conclusions are consistent with models proposed for other vacuolar ATPases (Arai *et al.*, 1989;1990a,b).

## 6.2. METHODS.

### 6.2.1. Neuraminidase and Glycopeptidase digestions.

For degradation with neuraminidase, chromaffin granule membranes (1 mg/ml) or the P1 fraction from Triton X-114 fractionation (0.25 mg/ml) obtained as indicated in Chapter 4, were incubated for three hours at 30°C in 0.2 M-Mes/NaOH buffer, pH 5.5, containing 1.6 mM benzamidine, 6 µg/ml pepstatin, 6 µg/ml leupeptin, 0.4 mM PMSF, 0.05 mM TLCK, 0.05 mM TPCK and 1 unit of neuraminidase/ml (Sigma type VI). For treatment with glycopeptidase F, the conditions were 4 hours at 30°C in 20 mM Hepes/NaOH, pH 7.4, containing 9 mM EDTA, 1% (v/v) 2-mercaptoethanol, 6 µg/ml pepstatin, 6 µg/ml leupeptin, 1.6 mM benzamidine and 33 units/ml of glycopeptidase F. Where indicated n-octyl-β-glucoside (1%(w/v) final concentration) or SDS (0.1%, w/v) was included. Treated samples were analysed by SDS-PAGE and gels were stained with silver (see Materials and Methods, Chapter 2).

### 6.2.2. BACH treatment.

Biotinamidocaproylhydrazide (BACH) was utilized in an adaptation of the method by O'Shannessey (O'Shannessey and Quarles, 1985; O'Shannessey *et al.*, 1987). Briefly, chromaffin-granule membranes and reconstituted ATPase (obtained as in Chapter 4) were subjected to SDS-PAGE and transferred to nitrocellulose sheets as indicated in the Materials and Methods Chapter. The blot was incubated with 10 mM NaIO<sub>4</sub> in 0.1 M sodium acetate buffer, pH 5.5 in the dark; after washing (3 x 10 min) with phosphate/salt buffer (50 mM KH<sub>2</sub>PO<sub>4</sub>/KOH pH 6.5, 0.15 M NaCl), the blot was incubated with 2 mg of BACH in 10 ml of sodium acetate buffer for 1 hour and washed again in the same conditions as above. The nitrocellulose sheet was then blocked with 0.25% Tween 20 (1 h), labelled with [<sup>125</sup>I]Streptavidin (10 counts/second/ml; specific radioactivity approx. 25 MBq/mg protein, incubated for 1 h), washed (3 x 10 minutes) with Tris/salt buffer (10 mM Tris-HCl pH 7.4, 0.15 M NaCl), dried and exposed for autoradiography at -70°C using an intensifying screen. A control experiment, in which there was no oxidation with NaIO<sub>4</sub>, was also performed.

### 6.2.3. [<sup>125</sup>I] TID treatment.

Chromaffin granule membranes (0.25 mg) or purified reconstituted ATPase I (0.2 mg), as indicated on Chapter 3 (with intrinsic lipids), were incubated with 5 MBq of [<sup>125</sup>I]-labelled TID (total volume 0.5 ml) for 15 min in the dark at room temperature, then illuminated for 20

min at 330 nm, in a 5 mm x 5mm quartz cuvette in the sample holder of a Perkin-Elmer fluorimeter. Labelled proteins were analysed by electrophoresis and autoradiography.

#### 6.2.4. Further purification of the chromaffin granule ATPase I

Continuous density gradients of 5-20% glycerol were poured in 10% methanol in working buffer (10 mM Hepes/NaOH pH 7.4, 0.15 M KCl, 0.1 mM EDTA and 1 mM DTT). 1 mg/ml of lipid mixture containing 34.2% PC and PE, 20.6% cholesterol and 11.1% PS and 0.9% n-octyl- $\beta$ -glucoside or taurodeoxycholate were also present throughout the gradient. Gradients were poured as indicated in the Materials and Methods section; volumes were normally 5 ml although some 11 ml gradients were also attempted; 400 or 700  $\mu$ l respectively of solubilized ATPase (see Chapter 4) were loaded onto the gradients and centrifuged at 45,000 r.p.m. for 5 hours in a Beckman SW.50.1 rotor (small gradients) or at 35,000 r.p.m. for 15 hours in a Beckman SW.41 rotor (big gradients). After centrifugation 0.5 ml samples were collected and assayed for ATP hydrolysis. A peak of ATPase activity was usually found in fractions 3-4 in both types of gradient (fraction 1 being at the bottom). Samples with maximal activity and mixtures of the latter with samples from other parts of the gradients were subjected to dialysis against 10% methanol in working buffer for around 40 hours with 4-6 changes of buffer.  $H^+$ -translocation was then assayed. 11-ml glycerol gradients were calibrated by loading 700  $\mu$ l of a mixture of proteins containing equal amounts of  $\beta$ -amylase (150 kDa), catalase (230 kDa), apoferritin (460 kDa) and thyroglobulin (670 kDa). The concentration of the protein mixture was 0.2 mg/ml. Samples from gradients were routinely analysed by SDS-PAGE and the gels stained with silver as indicated in Chapter 2.

#### 6.2.5. Gel scanning

Silver-stained gels were scanned in a Joyce-Loebl Chromoscan 3 apparatus. Peaks corresponding to the different subunits were integrated by a computer programme and stoichiometry calculated by dividing the value of the integral by the respective apparent molecular mass. In order to check whether silver stain was quantitative, different amounts of solubilized ATPase in 1.8% n-octyl- $\beta$ -glucoside (1, 2, 3, 4 and 5  $\mu$ g) were analysed by SDS-PAGE, the gels stained with silver and tracks were then scanned.

#### 6.2.6. Aminoacid Analysis.

An adaptation of the method of Arai *et al.* (1988) was applied. Pure solubilized ATPase in n-octyl- $\beta$ -glucoside or taurodeoxycholate (2-3 ml), as well as the solubilized hydrophobic domain (see Results) of the enzyme were precipitated with 10 % TCA in a final volume of about 10 ml. Precipitates were recovered by centrifugation at 15,000 r.p.m. for 30 min in a Beckman



10 ml. Precipitates were recovered by centrifugation at 15,000 r.p.m. for 30 min in a Beckman JA-20 rotor ( $g_{av} = 17,600$ ) and then delipidated by treatment with 10 ml of acetone:ethanol(1:1) on ice-salt (see Materials and Methods Chapter) for 1 hour, after which they were subjected to centrifugation under the same conditions as above. The final precipitates were resuspended in 1 ml of distilled water and lyophilized. After lyophilization samples were dissolved in 30  $\mu$ l of 1% SDS in 10 mM Hepes-NaOH pH 7.

Concentrated delipidated samples were analysed by SDS-PAGE using a Bio-Rad "Tall mighty small" slab-gel equipment, transferred to Immobilon and the blot stained with Coomassie-Blue, as described in the Materials and Methods section. Destaining was performed in 50% methanol, after which the blot was dried and individual polypeptides were cut out and subjected to aminoacid analysis in an Applied Biosystems apparatus.

To determine the efficiency of blotting for each polypeptide, another sample of pure ATPase was labelled with the water-soluble Bolton-Hunter reagent ( $[^{125}\text{I}]\text{S-SHPP}$ ). This was prepared according to the method of Thompson *et al.* (1987): 0.25 mCi  $\text{Na}^{125}\text{I}$  in 10  $\mu$ l of phosphate buffer, 5  $\mu$ l of 17.8 mM chloramine T in phosphate buffer, 50  $\mu$ l of 5 mM hydroxyphenylacetic acid in water and 5  $\mu$ l of 63 mM sodium metabisulphite also in phosphate buffer were added sequentially in that order to 10  $\mu$ l of 0.55 mM S-SHPP in DMSO.

20  $\mu$ l of each sample were mixed with 10  $\mu$ l of  $[^{125}\text{I}]\text{S-SHPP}$ . The mixture was incubated on ice for 30 min. The material was treated with sample buffer, split in two and analysed by SDS-PAGE, along with unlabelled material, as described above. One of the tracks with labelled protein was not transferred, the gel being stained with Coomassie blue and subjected to densitometric scanning. The other track was transferred to Immobilon and stained. The different labelled bands were cut from the gel and the blot and the radioactivities were quantitated in a  $\gamma$ -counter. The fractional efficiency of transfer was calculated by dividing the radioactivity of each band in the blot by that found in the gel.

#### 6.2.7. Immunological labelling of samples from different stages of the fractionation of chromaffin granule membranes with Triton X-114.

In order to find out whether some subunits of the chromaffin granule ATPase separated from the enzymic complex during the treatment of membranes with Triton X-114, as suggested by analysis of fractions collected from glycerol gradients (see Results and Discussion), immunolabelling experiments were performed on the supernatant remaining after removal of P1 during the fractionation of chromaffin granule membranes with Triton X-114.

Fractions were obtained as described in the Materials and Methods chapter, about 50  $\mu$ g of each were then lyophilized and resuspended in 150  $\mu$ l of sample buffer. Three 8 x 8 cm gels were run with 5  $\mu$ g of samples in each track. Gels were then transferred to nitrocellulose sheets as indicated in Chapter 2. Blots were stained with Ponceau S to check the transferring and then



destained with 10mM Tris-HCl/150 mM NaCl, pH 7.4 (Tris/salt buffer); then they were blocked with 0.5% Tween 20 in Tris/salt buffer. Nitrocellulose sheets were incubated overnight with antibodies against the 72 kDa and 57 kDa subunits from *Kalanchoe Daigremontiana* vacuolar ATPase, and against the 120 kDa subunit from chromaffin granule ATPase, respectively. Antisera were diluted 100-fold in Tris/salt buffer containing 30 mg/ml BSA and 5% (v/v) heat-treated horse serum.

After overnight incubation, blots were washed with Tris/salt buffer (2 x 5 min), 0.1% Tween 20 in Tris/salt buffer (5 min) and Tris/salt buffer again (5 min), then they were incubated for 1 hour with anti-rabbit IgG antibody, peroxidase conjugate (5,000 times dilution in 1 mg/ml BSA in Tris/salt buffer) after which they were washed as above. Labelled bands were detected using an ECL Western Blotting detection system.

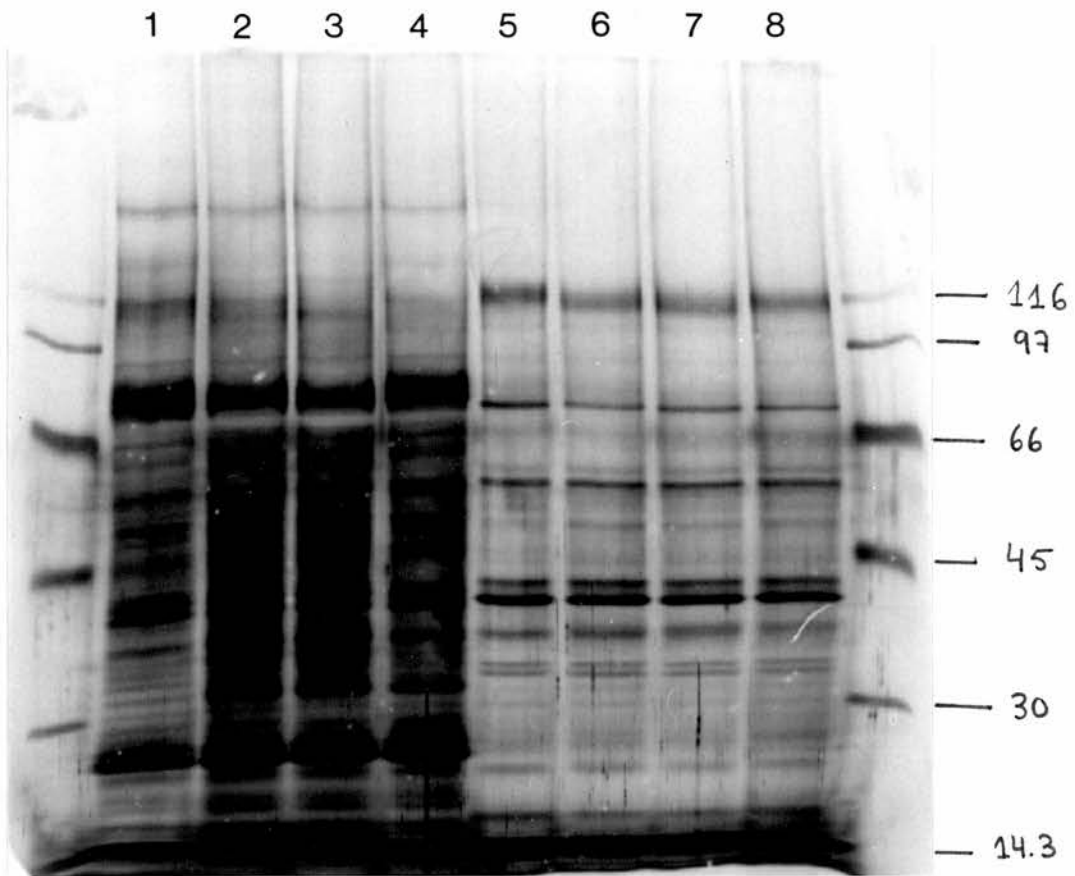
### 6.3. RESULTS.

#### 6.3.1. Neuraminidase and Glycopeptidase F digestions.

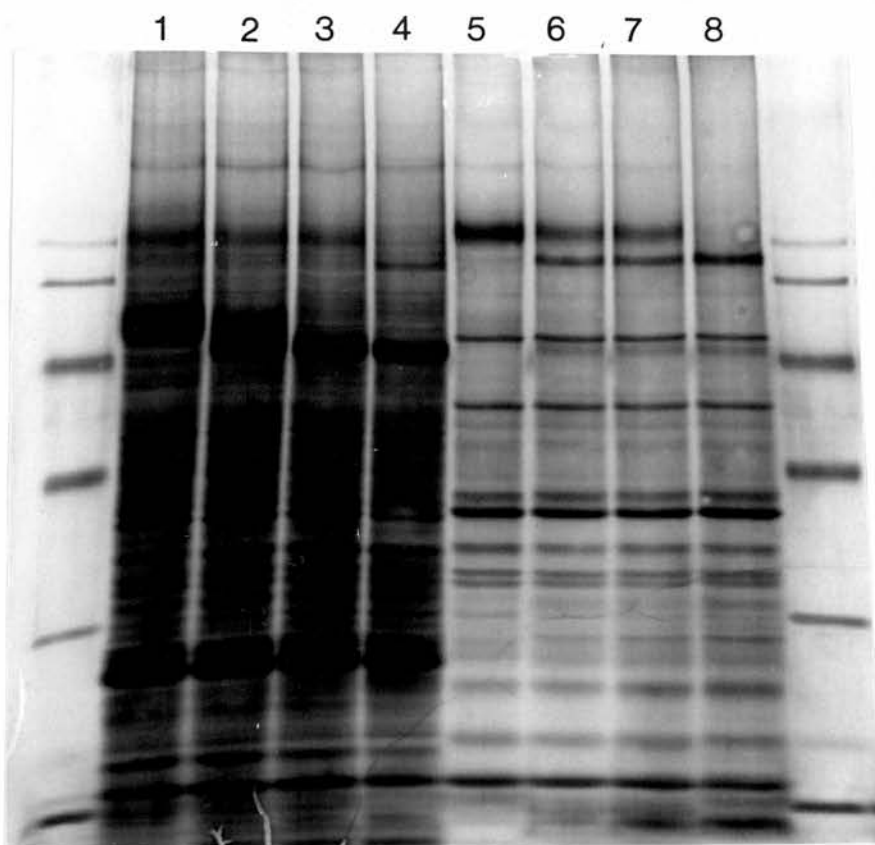
The results of enzymic deglycosylation are shown in figures 6.1, 6.2 and 6.3. Of the putative ATPase I polypeptides only one, the 120 kDa subunit, was affected. Neuraminidase treatment produced a slight reduction in its apparent molecular mass (Figures 6.1 and 6.3). More convincing is the significant reduction in molecular mass (around 10 kDa) produced by glycopeptidase F (Figures 6.2 and 6.3). The faint band of apparent molecular mass of 71 kDa that appears on glycopeptidase F treatment (Figures 6.2 and 6.3, tracks 6-8) is the deglycosylated form of dopamine- $\beta$ -hydroxylase, present as a contaminant in the P1 fraction; it is seen more clearly in tracks 3 and 4.

#### 6.3.2. Treatment with BACH.

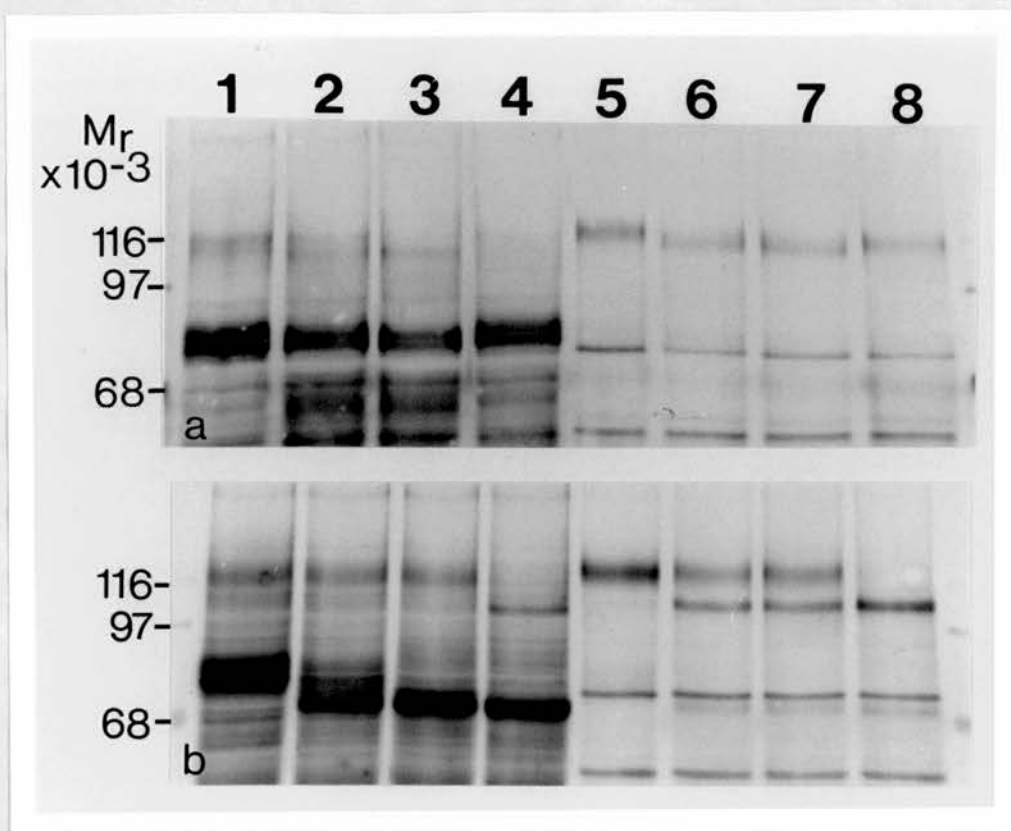
The result of a typical BACH-labelling experiment is shown in figure 6.4(a) which is a densitometric scan of an autoradiograph. It can be observed that there is a big background in the membrane track, due to the abundance of glycoproteins in it. The biggest peak is that of DBH which is the major protein in the membranes and is glycosylated. In the purified ATPase the DBH peak is significantly reduced and the background disappears; a new peak can be observed at 120 kDa which corresponds to subunit I of the ATPase. The purified ATPase fraction is also enriched in bands at 18 and 16 kDa approximately but these two polypeptides also appear in a control that had not been treated with NaIO<sub>4</sub> (figure 6.4(b)) which means that they are non-specifically labelled. It is also notable that a glycosylated polypeptide of about 47 kDa copurifies with the ATPase.



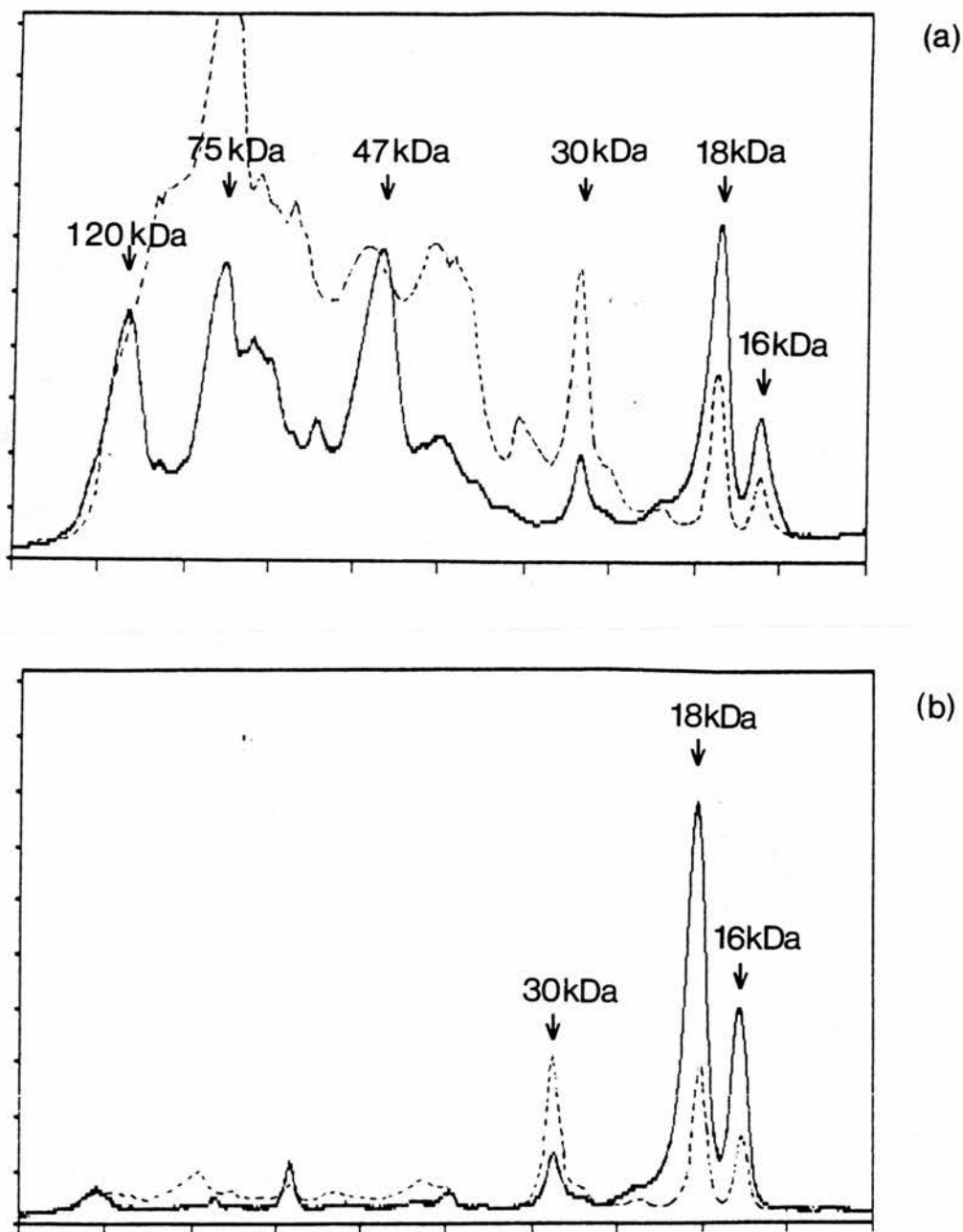
**Figure 6.1. Degradation of oligosaccharide chains with Neuraminidase.** Tracks 1-4, chromaffin granule membranes; tracks 5-8, P1 fraction. Tracks 1 and 5, controls (no enzyme); 2 and 6, digested without detergent; 3 and 7, with 1% (w/v) n-octyl- $\beta$ -glucoside; 4 and 8, with 0.1% (w/v) SDS. Outer tracks, molecular mass standards [from top to bottom:  $\beta$ -galactosidase (116 kDa), phosphorylase b (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa)]. Stain was with silver



**Figure 6.2. Degradation of oligosaccharide chains with Glycopeptidase-F.** Tracks 1-4, chromaffin granule membranes; tracks 5-8, P1 fraction. Tracks 1 and 5, controls (no enzyme); 2 and 6, digested without detergent; 3 and 7, with 1% (w/v) n-octyl- $\beta$ -glucoside; 4 and 8, with 0.1% (w/v) SDS. Outer tracks, molecular mass standards as on Figure 6.1.



**Figure 6.3.** Upper parts of gels shown in figures 6.1 and 6.2.



**Figure 6.4. Labelling of glycoproteins with BACH: densitometric scan of autoradiograph.** (a) Samples subjected to complete treatment; (b) samples not subjected to oxidation with  $\text{NaIO}_4$  (see text for details). Dotted lines represent chromaffin granule membranes and continuous lines purified, reconstituted ATPase.

### 6.3.3. Treatment with $^{125}\text{I}$ TID.

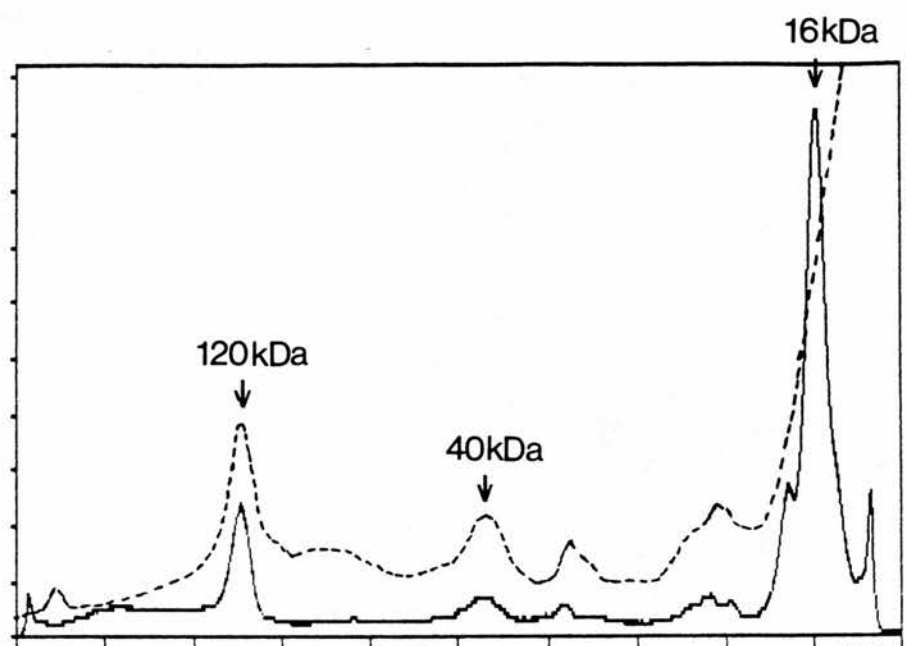
The results of this experiment are shown in figure 6.5, which is a densitometric scan of an autoradiograph of labelled samples analysed by SDS-PAGE. In chromaffin granule membranes the probe labels the 120 and 16 kDa subunits of the ATPase, which are the most intensely labelled of all polypeptides in the membrane and, as expected, cytochrome  $b_{561}$  is also labelled. It is notable that the 16 kDa subunit is not resolved from other components of low molecular mass, which are probably lipids. With purified, reconstituted ATPase, the results are more clear-cut; the 120 and 16 kDa subunits are the only intensely labelled polypeptides, however, it is worth pointing out that a polypeptide of about 40 kDa is also slightly labelled.

### 6.3.4. Further purification of the $\text{H}^+$ -ATPase.

Figures 6.6(a) and (b) show SDS-PAGE analysis of samples collected from the gradients containing n-octyl- $\beta$ -glucoside or taurodeoxycholate respectively. It can be seen that fractions 6-8 in both cases show subunits at 120, 72, 57, 43, 40, 33, 18 and 16 kDa. ATPase activity is found here (see figure 6.6(c)), with peak specific activities of 2-2.5 units/mg in the case of n-octyl- $\beta$ -glucoside and 4 units/mg in the case of taurodeoxycholate. A small quench was observed when these samples were tested for  $\text{H}^+$ -translocation after dialysis using the ACMA assay. Further up in the gradients, strong bands corresponding to polypeptides of molecular masses 120, 40, 18 and 16 kDa copurify together. No  $\text{H}^+$ -translocation or ATP hydrolysis were observed in these samples. Attempts to mix fractions from different regions of the gradients did not produce any enhancement of any of the activities, only a consistent dilution of both in those fractions containing all subunits. Increased resolution was attempted by lengthening the time of centrifugation to 7 hours or by using 11-ml glycerol gradients (centrifuged for 15 h). SDS-PAGE analysis of fractions collected in these two cases are shown in figures 6.7(a) and 6.8(a) respectively: it can be seen that the same two groups of polypeptides appear separated to about the same extent as before. Figures 6.7(b) and 6.8(b) show the distribution of ATPase activity in these cases, and in the latter case the position of marker proteins of different molecular masses are shown. Figure 6.9 further shows a representation of sedimentation constant vs. fraction number for a 11-ml glycerol gradient.

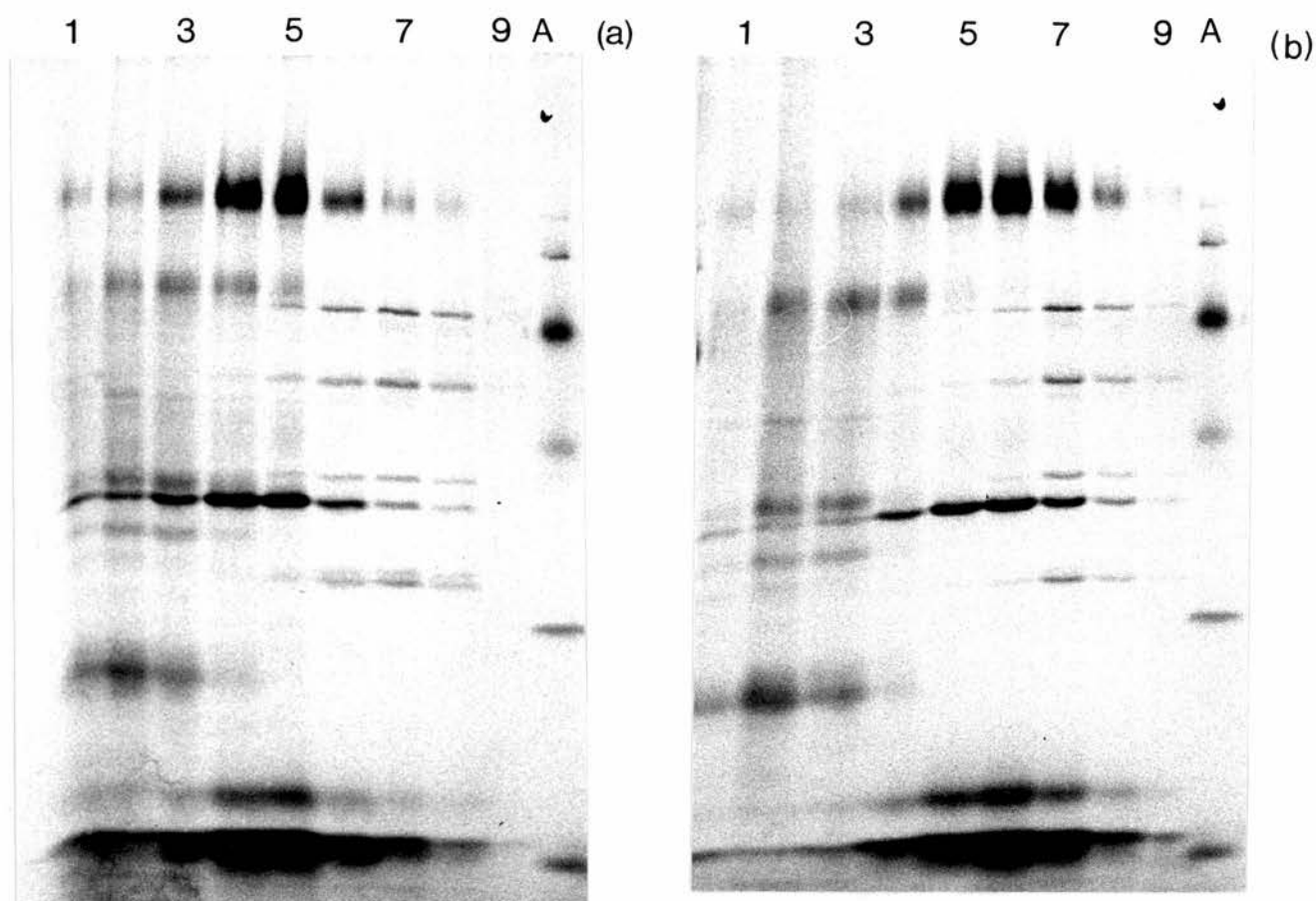
### 6.3.5. Gel scanning.

Figure 6.10 shows a graph of integral against amount of protein loaded for different bands of the chromaffin granule ATPase. Table 6.1 shows the stoichiometries calculated by densitometric scan of different samples from the gradients analysed by SDS-PAGE and stained with silver and Coomassie blue.

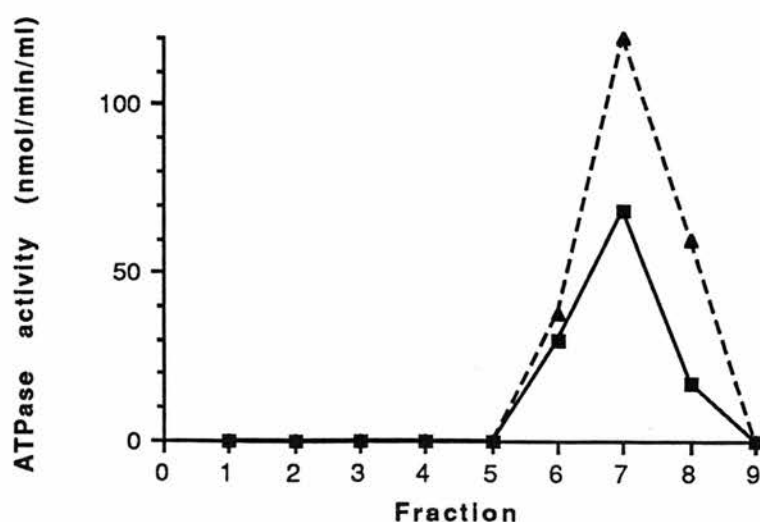


**Figure 6.5. Photolabelling with  $[^{125}\text{I}]\text{TID}$ : densitometric scan of autoradiograph.** Dotted line, chromaffin granule membranes; continuous line, purified reconstituted ATPase.

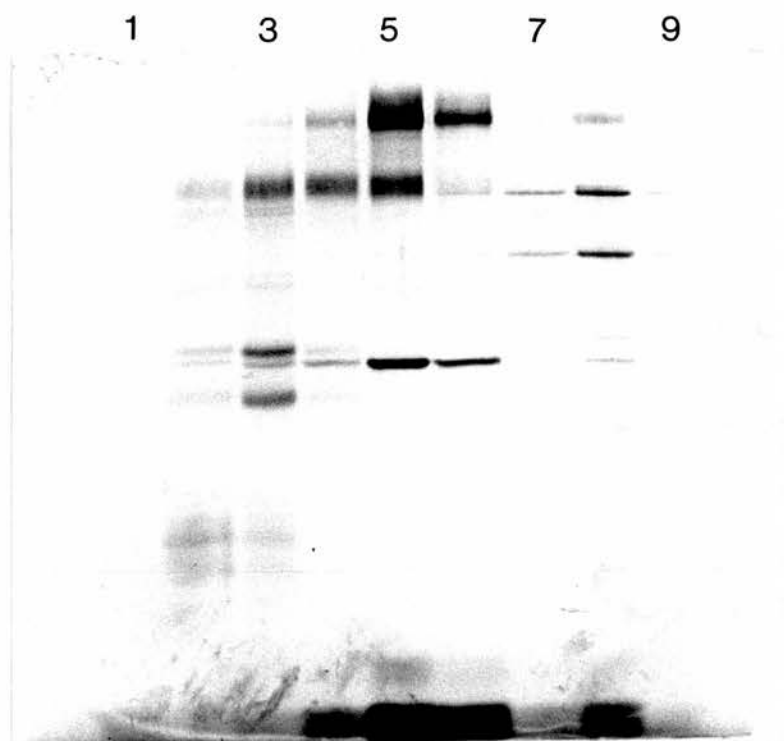




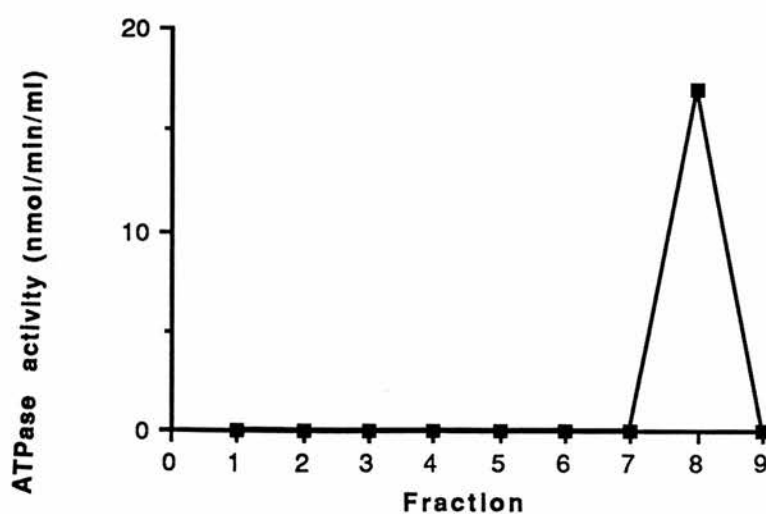
**Figure 6.6.** SDS-PAGE analysis of fractions collected from 5 ml glycerol gradients containing n-octyl- $\beta$ -glucoside (a) and taurodeoxycholate (b). Gradients were run for 5 hours as described in text. Tracks 1-9 in each figure, fractions collected after ultracentrifugation of solubilized ATPase on 5-20% glycerol gradients. Fraction 1 is top of the gradient and fraction 9 bottom. About 30  $\mu$ l of sample were loaded in each track. Stain was with silver. Tracks A, molecular mass standards as in figure 6.1.



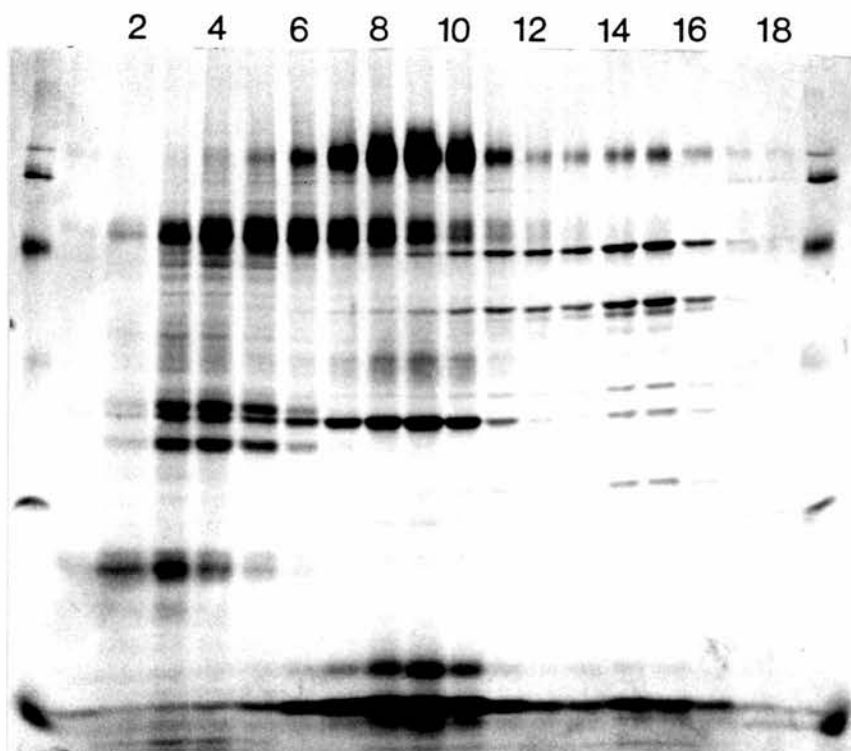
**Figure 6.6(c).** Distribution of ATPase activity in gradients shown in figure 6.6(a) (■ --- ■) and 6.6(b) (▲ --- ▲).



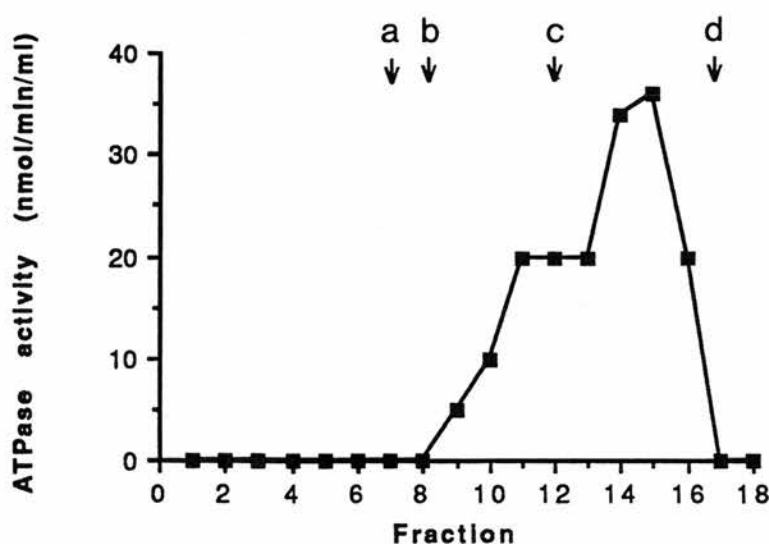
**Figure 6.7(a).** SDS-PAGE analysis of fractions collected from 5 ml glycerol gradients containing n-octyl- $\beta$ -glucoside. A 10% straight gel was run in this case. Gradients were run for 7 hours. Tracks 1-9, collected fractions. Other conditions were as in figure 6.6.



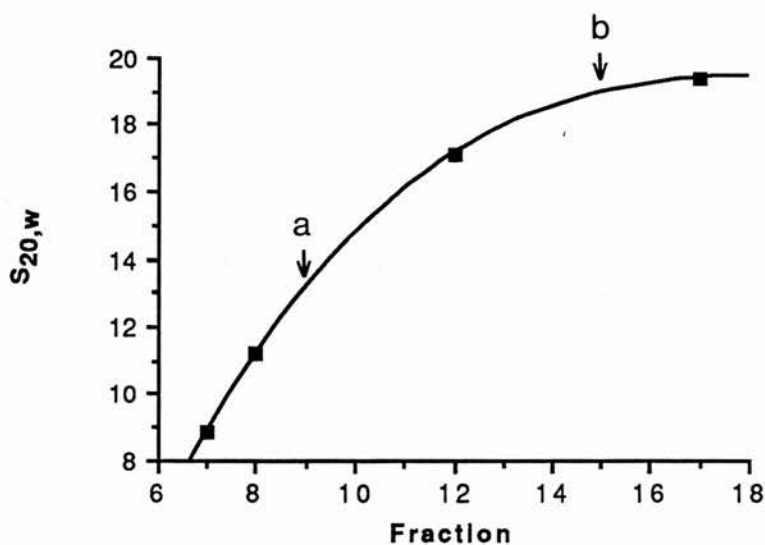
**Figure 6.7(b).** Distribution of ATPase activity in gradient shown in figure 6.7(a).



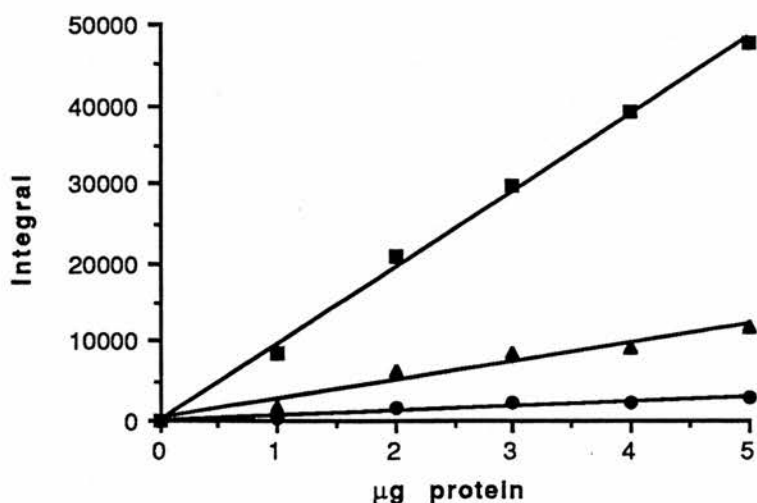
**Figure 6.8(a).** SDS-PAGE analysis of fractions collected from 11 ml glycerol gradients containing n-octyl- $\beta$ -glucoside. Gradients were run for 15 hours as described in text. Tracks 1–18, collected fractions. Fraction 1 is top of the gradient and fraction 18 bottom. About 30  $\mu$ l of sample were loaded in each track. Stain was with silver. Outer tracks, molecular mass standards as in Figure 6.1.



**Figure 6.8(b).** Distribution of ATPase activity in gradient shown in figure 6.8(a). Arrows indicate where the peaks of the calibrating proteins were found: (a)  $\beta$ -amylase (200 kDa); (b) Catalase (240 kDa); (c) apoferritin (450 kDa); (d) thyroglobuline (670 kDa).



**Figure 6.9 . Representation of the sedimentation coefficient vs. fraction in gel shown in figure 6.8(a).** The calculated sedimentation coefficients for the complete enzyme (b) and the hydrophobic domain (a) are 19.0 and 13.1 respectively.



**Figure 6.10. Proportionality between intensity of silver-staining and amount of protein.** Different amounts of solubilized ATPase were analysed by SDS-PAGE and the gels stained with silver, as described in the Materials and Methods Chapter. Gels were scanned and peaks corresponding to the different bands were integrated as indicated in text. Figure shows a plot of the values of the integral of silver-staining density vs. the amount of analysed protein for the 120 (▲) and 40 kDa (●) subunits of the chromaffin granule ATPase and for the overall integral calculated in each track (■).

**Silver-stained gels**

	Subunit							
	120	72	57	43	40	33	18	16
<b>n-octyl-<math>\beta</math>-glucoside:</b>								
- Holo-enzyme	24 (1)	70 (3)	71 (3)	21 (1)	30 (1)	29 (1)	-	312 (13)
- Hydrophobic domain	106 (1)	-	-	-	122 (1)	-	161 (1)	638 (5)

**Taurodeoxycholate:**

- Holo-enzyme	146 (5)	36 (1)	42 (1)	28 (1)	142 (5)	65 (2)	481 (13)	1229 (34)
- Hydrophobic domain	0.23 (1)	-	-	-	0.23 (1)	-	0.23 (1)	1.1 (5)

**Coomassie blue-stained gels**

- Holo-enzyme	1.3 (1)	19(15)	23 (18)	-	-	-	-	28.4 (22)
- Hydrophobic domain	10 (1)	-	-	-	-	-	-	68.5(7)

**Table 6.1. Stoichiometries for chromaffin granule ATPase and the hydrophobic domain calculated from densitometric scanning of gels.** Numbers indicate value of integrals divided by molecular masses in kDa. Molar ratios (bold figures in parenthesis) were calculated by dividing these values by the number giving ratios closest to integral values.

#### 6.3.6. Aminoacid analysis

Results are shown in Table 6.2. The stoichiometry was calculated as follows (Table 6.3): the total number of moles of each aminoacid for every band was calculated and corrected according to the efficiency of transfer. This figure was multiplied by the average molecular mass of aminoacids in each polypeptide, calculated according to the percentage of each aminoacid present. This number was then divided by the molecular mass of each band estimated from their respective migration on SDS gels. The numbers thus obtained were divided by the smallest one and approximated to the nearest integral values.

#### 6.3.7. Immunolabelling experiments.

Figures 6.11a, b and c show autoradiograph of the blots developed with ECL. It can be seen that the 120, 72 and 57 kDa subunits appear always in the same fractions: chromaffin granule membranes, P1 and the supernatant from the washing of the P1 with Triton X-114.

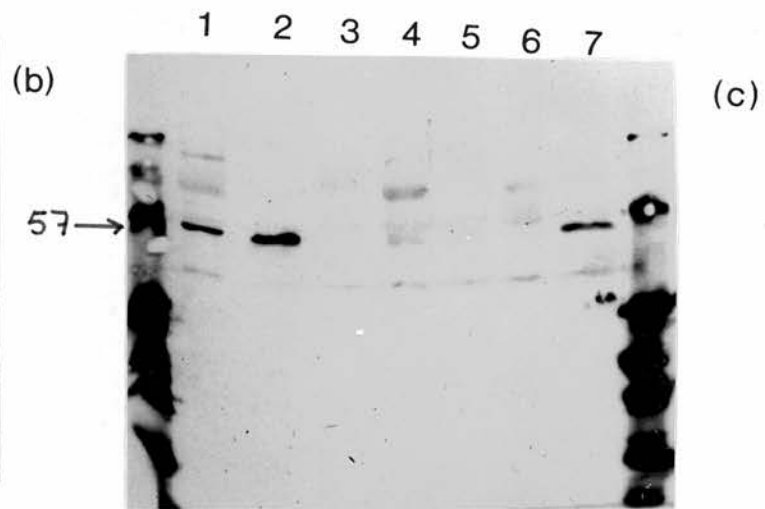
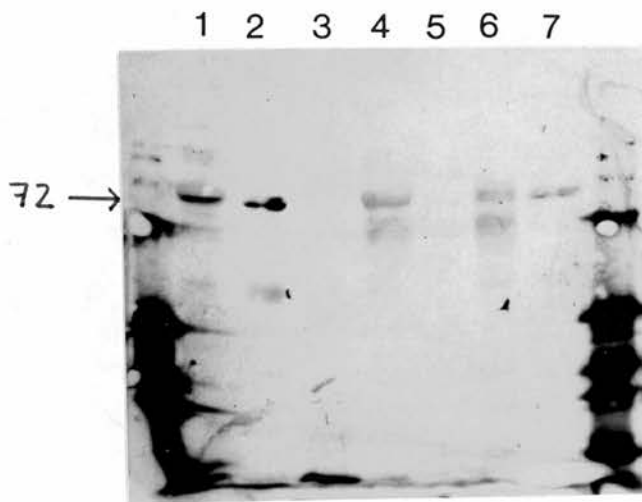
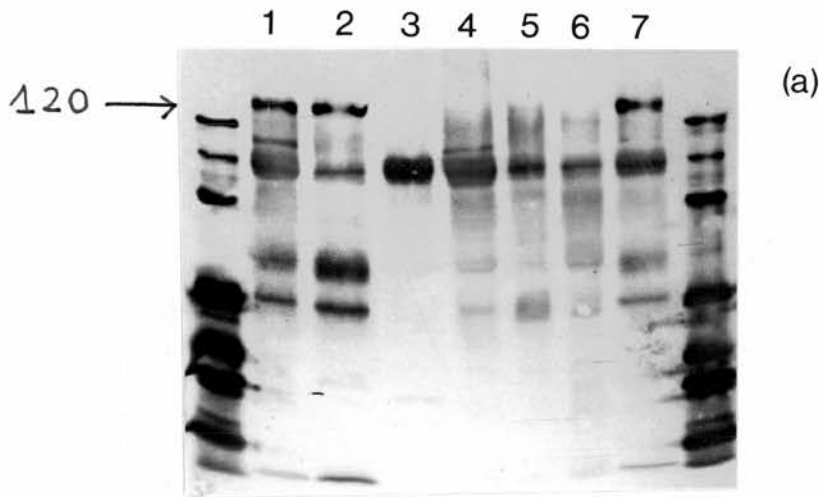
	Subunit Molecular Mass (kDa)							
	120	120 (*)	72	57	43	33	16	16 (*)
Asp	5	6.8	6.6	8.3	--	--	5.4	6.7
Glu	9.8	10.2	10.4	9	13.8	21.5	7.4	9.7
Ser	9.8	11	5.7	9.35	7.2	--	10.9	13
Gly	30.7	23.5	12.1	21.3	19	20.9	23.5	33.3
Arg	3.9	5.2	6.4	6.5	--	--	4.9	--
Thr	3.2	3.2	3.4	4.1	--	--	4.9	--
Ala	6.7	7.8	11	8.5	12.3	10.3	9.9	11.4
Pro	--	2.5	2	1.7	--	--	--	--
Tyr	2.6	2.2	2.6	2.3	--	--	2.8	--
Val	6.2	7.8	12	6.5	16.3	14.6	9	11.5
Ile	6.9	2.5	5.6	3.7	16.7	22	3	7.5
Leu	9.3	9.3	13.5	9.4	14.8	10.7	10.3	--
Phe	2.6	3.3	3.8	2.8	--	--	3.6	--
Lys	3.2	4.6	4.9	5	--	--	4.4	6.8

**Table 6.2. Aminoacid composition of the subunits of the chromaffin granule ATPase.** Values are mole percentages and represent the result of a single analysis. Aminoacid compositions were determined for complete enzyme and hydrophobic domain (\*) purified by ultracentrifugation through 5-20% glycerol gradients containing n-octyl- $\beta$ -glucoside. Polypeptides of 40 and 18 kDa subunits did not stain with Coomassie and were not analysed. Samples obtained from gradients containing taurodeoxycholate were not precipitated properly by treatment with TCA and acetone:ethanol, as a consequence not enough protein could be recovered in the delipidation/concentration step to perform the analysis (see text for details).



Subunit (kDa)	Total aminoacids (pmol)	Total protein (pmol)	Uncorr. Stolch.	Blotting Efficiency (%)	Corrected Stolch.
120	193.79	0.185	1	13	2
72	188.76	0.324	2	53.5	1
57	348.52	0.727	4	56.4	2
43	57.61	0.152	1	45	1
33	54.77	0.193	1	28.5	1
16	150.7	1.090	6	24.6	6
120(*)	385.12	0.374	1	66	1
16(*)	58.92	0.387	1	37	2

**Table 6.3. Subunit stoichiometry of the chromaffin granule ATPase calculated from the aminoacid analysis of complete enzyme and hydrophobic domain (\*) purified by ultracentrifugation through density gradients in glycerol containing n-octyl- $\beta$ -glucoside.**



**Figure 6.11 Immunolabelling of different fractions of the fractionation of chromaffin granule membranes with antibodies against the 120 kDa (a) subunit of chromaffin granule  $H^+$ -ATPase and the 72 (b) and 57 (c) kDa subunits of *Kalanchoe daigremontiana*  $H^+$ -ATPase.** Track 1, chromaffin granule membranes; Track, 2, P1; track 3, detergent rich phase; track 4, aqueous phase; track 5, glycoprotein fraction; track 6, post-aqueous supernatant; track 7, supernatant resulting after washing P1 with 2% Triton X-114; outer tracks, set of protein A-fusion proteins of known molecular mass [from top to bottom: 95, 73, 51, 40, 31, 24 and 18 kDa]

## 6.4. DISCUSSION.

The results reported in this Chapter demonstrate that the 120 kDa subunit of chromaffin granule ATPase I is glycosylated. A glycoprotein of around 47 kDa copurifies with the enzyme up to this stage of the purification, however, it separates from the enzymic complex when the solubilized ATPase is subjected to ultracentrifugation in density gradients (see figure 6.8(a)) which suggests that it is not a component of the enzyme. The fact that the 120 kDa subunit was strongly labelled by the hydrophobic probe TID further suggests that it is an integral membrane protein.

Hüber *et al.* (1979) demonstrated that the major glycoproteins in chromaffin granules have their oligosaccharide chains located exclusively on the extracytoplasmic face; these results, along with others obtained in our laboratory that show that this subunit can be radioiodinated, biotinylated and proteolysed in intact granules and is not solubilized at pH 11, further suggest that the 120 kDa subunit is a transmembrane polypeptide whose polysaccharide chains are located within the granule matrix. This is consistent with the structure predicted by hydrophobicity analysis of the amino acid sequence (Perin *et al.*, 1991).

Results with the hydrophobic probe TID also confirmed that the 16 kDa subunit is an integral membrane polypeptide, and the 40 kDa subunit might have a small part in the membrane.

Glycerol gradients yield a pure and highly active form of chromaffin granule ATPase I, thus demonstrating the power of this technique, already mentioned in Chapters 3 and 4, in purifying enzymes of this type. It is also noteworthy that enzyme purified in the presence of taurodeoxycholate showed maximal ATP hydrolytic activity although, when the purification was performed in *n*-octyl- $\beta$ -glucoside, better  $H^+$ -translocation was observed. The latter fact is very likely to be due to the intrinsic characteristics of *n*-octyl- $\beta$ -glucoside that make it easier to remove by dialysis. Taurodeoxycholate had earlier been applied to the purification of the ATPase I from chromaffin granules by Apps *et al.* (1983).

The most striking result from the glycerol gradients is the separation of two complexes, one containing polypeptides of molecular masses 120, 72, 57, 43, 40, 33, 18 and 16 kDa and the other of 120, 40, 18 and 16 kDa. The first of these complexes was associated with the peak in ATPase and  $H^+$ -translocation activities. By calibration of the gradients with proteins of known molecular masses, it was estimated that the small complex had a molecular mass of around 200-230 kDa whereas the entire complex had an estimated molecular mass of around 600 kDa.

A further investigation of this result showed that no further resolution of the first complex could be achieved by lengthening the time of centrifugation or using density gradients with higher resolution, that is, it seems to be a single complex. This suggests that more polypeptides than previously thought may be associated with the enzymic activities which would imply that

the enzyme is more similar to its counterpart from clathrin-coated vesicles than has been reported so far. It is of course likely that the H<sup>+</sup>-ATPase in brain clathrin-coated vesicles originates in neurosecretory granules.

A possible explanation for the presence of a complex formed by subunits of 120, 40, 18 and 16 kDa is that these subunits actually form a hydrophobic domain which, as shown by studies with TID, would be almost completely embedded in the membrane. Treatment with Triton X-114 produces a separation of proteins according to their hydrophobicity, and if the enzyme has two domains it is possible that, in the initial treatment with detergent, a fraction of the ATPase I passes into the pellet of hydrophobic proteins as an intact complex and part is dissociated into these two domains, one going into the pellet, in common with other membrane-embedded subunits, and the more hydrophilic complex containing the 72, 57, 43 and 33 kDa subunits, into the supernatant. When the resolubilized pellet is subjected to density gradient centrifugation the holo-enzyme, which has a greater sedimentation rate, separates from the hydrophobic domain. This explanation implies that an excess of the hydrophilic subunits should be found in the Triton X-114 supernatant.

Immune blots carried out on the different phases from the Triton X-114 supernatant with antibodies raised against the 72 and 57 kDa subunits from the ATPase from *Kalanchoe Daigremontiana* and against the 120 kDa subunit from the chromaffin granule ATPase have, however, shown no excess of the 72 or 57 kDa subunits in any of the other fractions of the chromaffin granule membrane, the three subunits appearing always together. This could mean that the loss of the extrinsic domain of the enzyme could have occurred during the purification of the membranes from adrenal medullae, however this was not investigated. Another possibility is that the hydrophobic domain actually exists on its own within the granule membrane acting as an independent ion channel.

The existence of two separable domains would give strong support to the idea of these enzymes having a similar structure to that of F-type ATPases and thus a strong evolutionary relationship. In order to obtain further proof of this relationship, the stoichiometry of the enzymic complex was calculated.

The stoichiometries calculated by densitometric scanning of silver-stained gels for enzyme purified by ultracentrifugation through glycerol gradients containing taurodeoxycholate differs quite dramatically from that calculated for the enzyme obtained from n-octyl- $\beta$ -glucoside-containing gradient; the latter results being consistent with results obtained by Arai *et al.* (1988). This suggests that the separation of the complete complex from the hydrophobic domain is best achieved in the presence of n-octyl- $\beta$ -glucoside. The main inconsistency of these results is the unacceptably high values of number of the 18 and 16 kDa subunits per molecule of protein, however, when the stoichiometry of the hydrophobic domain is calculated, values are more reasonable. When Coomassie blue-stained gels were subjected to densitometric scanning, the stoichiometry found for the hydrophobic complex (120 kDa subunit:16 kDa subunit, 1:7) is also more reasonable than that found for the holo-enzyme,

where the hydrophobic subunits seemed to be understained with respect to the 72 and 57 kDa subunits; however, these results confirm that the molar ratio between the latter polypeptides is 1:1. In any case, it has to be considered that, although gel-staining may be proportional to the amount of a particular protein present this does not mean that all proteins are stained to the same extent, and that apparent molecular weights calculated from mobilities in SDS-PAGE may be quite inaccurate; therefore, these results can be taken no more than as a rough estimate of the stoichiometry of the complex.

A more accurate calculation of the stoichiometry of the chromaffin granule ATPase was attempted by quantitative aminoacid analysis. Unfortunately the bands corresponding to the 40 and 18 kDa subunits could not be located by Coomassie staining, so their analyses were not performed. The results obtained did not allow us to draw clear conclusions. The main problems regarding aminoacid analysis can be observed in Tables 6.2 and 6.3. First, there is a poor correlation between the aminoacid composition of the 120 and 16 kDa subunits from the complete enzyme and the hydrophobic domain, especially for the 16 kDa subunit. It could be argued that the so-called hydrophobic domain may be an independent complex and, thus, a different protein which would have a different sequence; however, this seems unlikely as shown by SDS-PAGE analysis of density gradient fractions (figures 6.6–6.8); second, although the uncorrected stoichiometry of the enzymic complex (that is, without taking into account blotting efficiency) looks reasonable, the corrected values are strongly affected by the apparent low efficiency of transfer for the 120 kDa subunit of the entire complex (13%). This latter fact is also inconsistent with the transfer efficiency of the same subunit of the hydrophobic domain (66%). The molar ratio 120 kDa subunit:16 kDa subunit in the hydrophobic domain (1:2) is different to that calculated for the entire complex (1:3), although this may not be very significant; in any case, the ratios are smaller than those found in the literature and those obtained by gel-staining.

The most likely explanation for the inconsistencies observed is that the total amount of protein analysed is minute, being two orders of magnitude smaller than the amount used by other groups (Arai *et al.*, 1988) so that the less abundant aminoacids could not be analysed and the percentages of the rest were therefore affected. This problem was particularly serious for the 16 kDa subunit of the hydrophobic complex, the amount of aminoacid analysed being only 59 pmoles against 151 pmoles analysed for the 16 kDa subunit of the complete enzyme (see table 6.2). This meant that a larger number of aminoacids did not appear in the analysis of the former (see table 6.2), thus producing very different apparent compositions. Another noticeable result from the aminoacid analysis is the extremely high percentage of glycine found. This may be due to the electrode buffer used in the SDS-PAGE analysis (Tris/glycine, see Material and Methods). This contamination, although small in absolute terms, may interfere quite strongly with the analysis performed in this case because of the low amount of protein analysed.

As far as blotting efficiency is concerned, the already-mentioned inconsistency between the percentage of transfer for the 120 kDa subunit of the entire complex and the same subunit of the



hydrophobic domain is difficult to explain. However, it has to be considered that gradients contain a high concentration of lipids (1 mg/ml) and, even though samples are treated with acetone:ethanol, this procedure is unlikely to remove the entire lipid content. This means that the analysed samples may still have had a quite high lipid content and, considering the low protein concentration, a very high lipid:protein ratio. Lipids (PE and PS) are labelled by the water-soluble Bolton-Hunter reagent, which binds to amino groups, and contribute to an unknown extent to the radioactivity found in gels and blots, thus biasing the calculated blotting efficiency.

We can conclude from the experiments presented in this chapter that the chromaffin granule ATPase complex is of high molecular weight and that eight different polypeptides of molecular masses 120, 72, 57, 43, 40, 33, 18 and 16 kDa copurify with its activities when subjected to ultracentrifugation in density gradients. The 120 kDa subunit is a glycoprotein and can be labelled by hydrophobic probes along with the 16 kDa subunit and, to a lesser extent, the 40 kDa subunit. Interestingly, these three polypeptides, along with another of 18 kDa, seem to form a complex that can be separated from the enzyme by ultracentrifugation through glycerol gradients. This complex does not show any of the enzymic activities.

The calculation of the stoichiometry of the chromaffin granule  $H^+$ -ATPase was attempted. In order to accomplish this, a purer form of the enzyme was obtained by ultracentrifugation through density gradients in glycerol (5-20%). Initial estimates of the stoichiometry by densitometric scanning of pure enzyme and hydrophobic domain analysed by SDS-PAGE and stained with silver and Coomassie blue gave values consistent with those found in the literature (see Table 6.1); however, confirmation of these values by quantitative aminoacid analysis of the different polypeptides of the enzyme was not conclusive, serious inconsistencies appearing when this approach was utilized.

Finally, it is worthwhile pointing out that a more reasonable calculation of the stoichiometry of the chromaffin granule ATPase might be achieved by improving the yield of the purification procedure, thus using a larger amount of material for aminoacid analysis that would not only allow the experiments to be performed more easily but would also minimize errors in the analysis of aminoacid composition. It would be particularly interesting to purify the protein by dissolving chromaffin granule membranes directly with *n*-octyl- $\beta$ -glucoside or taurodeoxycholate and subjecting the solubilized material to ultracentrifugation through glycerol gradients under the conditions described above. Such an experiment would simplify the purification procedure, would overcome problems of loss of enzyme during the Triton X-114 treatment and would confirm whether the hydrophobic domain does actually exist on its own in the chromaffin granule membrane.

**Chapter 7**  
**CONCLUSIONS AND PERSPECTIVES**



## 7.1. CONCLUSIONS

The aim of this research project was the characterization of the proton pump located in the membranes of the secretory granules of adrenal medullary chromaffin cells, known as "chromaffin granules". This enzyme belongs to a recently-identified group of membrane-bound ATPases, known as vacuolar or V-type ATPases that are distinct from the other previously-recognized groups, the P-type ATPases and the F-type ATPases.

As a first and fundamental step towards the objectives of the project an appropriate method of purification was developed. The detergent Triton X-114 had been utilized previously to separate membrane proteins according to their hydrophobicities (Bordier, 1984) and successfully applied to chromaffin granule membranes (Pryde and Phillips, 1986): Triton X-114 solubilizes many chromaffin granule membrane proteins but fails to solubilize the most hydrophobic proteins of the membranes, these latter coming out of the solution as a white precipitate (P1) that could be separated by centrifugation. This pellet contained ATPase I (Percy *et al.*, 1985) although the specific activity at this stage is low.

Fractionation with Triton X-114 was used as a starting point for the purification method. The use of the non-ionic detergent n-octyl- $\beta$ -glucoside allowed a quite specific solubilization of the ATPase from P1; however, the enzyme in solution had poor stability. Methanol proved to be crucial for the stabilization of the enzyme both in solution and, subsequently, in liposomes. The presence of lipids, either derived from the granule membrane or added from commercial preparations, and removal of detergent by gel filtration produced a great increase in ATPase activity in both cases and made it possible to measure proton transport in the second case. The stability of the enzymic activities (always stored in 10% methanol) was very much improved after this reconstitution into liposomes. The following conclusions were drawn from these results: as has been found for many membrane-bound proteins, it did not have optimal activity in detergent solution but needed to interact with a lipid layer, and once this was achieved activity and stability were greatly improved; furthermore, in order to be able to measure a transmembrane proton gradient, it was crucial that the protein was integrated into a sealed vesicle where protons could be accumulated, thus creating a measurable difference with respect to the outside of the vesicle.

Acridine derivatives have been utilized before to measure transmembrane gradients of protons: they are fluorescent weak bases that are freely permeant through biological membranes in their neutral form. These molecules can be trapped within acidic vesicles because their protonated form is impermeant, and molecular stacking produces self-quenching, so the net effect is a quench in the fluorescence of the solution in which these vesicles are suspended. Schuldiner *et al.* (1972) showed that the distribution of the acridine derivatives across a given

membrane was directly related to the distribution of protons and devised an expression that related the extent of the recorded quench to the difference of pH inside and outside the vesicle. Later, Casadio (1991) developed an adapted expression to account for probe-membrane interactions in the case of ACMA, which fitted much better to experimental data obtained under a variety of conditions, however, it did not deviate significantly from the Schuldiner equation in the range of quench we were involved in.

When ATPase reconstituted with commercial lipids was suspended in a solution containing the acridine derivative ACMA, a fluorescence quench was produced. The quench was absolutely dependent on the presence of  $Mg^{2+}$ , ATP and valinomycin; the initial level of fluorescence was restored on addition of FCCP or nigericin. The dependence on MgATP strongly suggested that the enzyme was responsible for the creation of the proton gradient, valinomycin being crucial to charge equilibration across the vesicle membrane by permitting exit of  $K^+$  as  $H^+$  were pumped in. The roles of FCCP and nigericin further suggested that a proton gradient was responsible for the recorded quench.

By a re-titration experiment the fluorescence was shown to follow the Schuldiner equation which demonstrated that it was suitable for our purposes of measuring the proton gradient created by the chromaffin granule proton pump. The initial rate of quench was also shown to be proportional to the amount of protein (activity) present, and on these and theoretical grounds it was concluded that this value was a good measure of the  $H^+$ -translocation activity of the enzyme.

Once the purification method was developed and an appropriate activity assay was found, different lipid mixtures were tried in order to optimize both enzymic activities. The fact that the lipid mixture did not affect the ATPase activity a great deal but did affect the  $H^+$ -translocation suggested that the ATP-hydrolysing subunits may not be integral membrane proteins and that due to the fact that sealed vesicles are crucial to measure a proton gradient we were probably optimizing conditions for obtaining strong, sealed liposomes rather than studying intrinsic lipid-protein interactions. The latter idea was further supported by the role played by cholesterol, which did not affect ATPase activity at all but was absolutely crucial to produce a measurable proton gradient.

The purification-reconstitution method enabled us to obtain chromaffin granule ATPase in quite a pure form, completely separated from ATPase II, and in an environment similar to that found in the granule membrane; furthermore, the possibility of measuring the  $H^+$ -translocation activity by a rapid and continuous method such as ACMA quenching meant that a suitable system to carry out detailed kinetic studies on the enzyme was now available.

The kinetic and regulatory properties of the reconstituted ATPase were then studied. All purine nucleotides (ATP, ITP and GTP) are substrates for the enzyme but not pyrimidines (UTP and CTP).  $Mn^{2+}$ ,  $Ca^{2+}$ ,  $Co^{2+}$  and  $Ni^{2+}$  can substitute  $Mg^{2+}$  as co-substrates.  $Mn^{2+}$  in particular activates the enzyme by increasing  $V_{max}$  and decreasing  $K$  for the ATP-cation complex.  $H^+$ -translocation obeys Michaelis-Menten kinetics in the absence of inhibitors,

however, ATP hydrolysis shows at least two  $K_m$  values. The latter result has also been observed by other groups (Moriyama *et al.*, 1989) and suggested to be a consequence of the existence of several ATP-hydrolysing subunits that interact with each other. The possibilities of an artefact of the activity assay and/or of the presence of a contaminating ATP-hydrolysing activity cannot be completely ruled out although similar results have been observed using different ATP hydrolysis assays and purification procedures and, indeed, enzymes from different sources.

In the presence of inhibitors (ADP, GDP and IDP) the behaviour of the enzyme is quite different and cooperative effects appear. Direct fitting of the experimental data to the Hill equation by use of an optimization program showed that ADP affected both  $V_{max}$  and  $K$  of the enzyme whereas the effect of IDP and GDP is much clearer on the  $K$ ,  $V_{max}$  not being affected to a great extent in the latter cases. A model based on the allosteric theory by Monod *et al.* (1965) was developed in order to explain these results: the experimental data can be explained by assuming that the enzyme is a multimeric structure that exists in two states that differ in their activities (e.g., R active, T inactive), the substrate binds to both states with different affinities and the inhibitor binds significantly only to the inactive state. This means that binding of the inhibitor stabilizes the inactive state thus affecting the  $V_{max}$  and the overall affinity of the substrate for the complex ( $K$ ). The fact that the enzyme shows near-Michaelis-Menten kinetics in the absence of inhibitor is explained by assuming that the affinity of the substrate for the inactive state is small compared to that for the active state and that the allosteric equilibrium constant ( $L$ ) is small, so that the substrate on its own does not displace the R-T equilibrium, the effects derived from the existence of the inactive state (i.e., cooperativity) disappearing. The model also predicts that the number of binding sites for the inhibitor (1) is different to the number of binding sites for the substrate (2).

The kinetic and regulatory studies support the idea of the chromaffin granule ATPase I being a multimeric structure, probably with a high degree of internal symmetry.

Structural studies demonstrated the existence of a glycosylated subunit (120 kDa) in the complex. The significance of this is not very clear although it might be important for the enzyme-membrane interaction or in regulating the enzyme when it is inserted into the plasma membrane on exocytosis. Labelling studies with a hydrophobic probe showed that the 120 kDa subunit is a transmembrane protein and that the 16 kDa subunit is embedded in the granule membrane. A small degree of labelling can also be observed in the 40 kDa subunit suggesting that this protein is mainly cytoplasmic although it probably has a small membrane-bound part. The results obtained with the hydrophobic probe are interestingly related to the distribution of the enzyme subunits when partially-purified enzyme was subjected to centrifugation through glycerol density gradients: a very active form of the enzyme that shows eight bands when analysed by SDS-PAGE is separated from an inactive complex formed by four different polypeptides. The respective apparent molecular masses of the polypeptides are 120, 72, 57,

43, 40, 33, 19 and 16 kDa in the first case and 120, 40, 19 and 16 kDa in the second. It could be observed that the subunits labelled by the hydrophobic probe were separated from the complete enzyme suggesting that the enzyme has two separable domains with different hydrophobicities. However, immunological studies showed that the separation between these two domains did not occur during the purification of the protein from chromaffin granule membranes. This suggested either that the hydrophilic domain separates from the membrane during the isolation of the latter from adrenal medullae or that the hydrophobic domain can exist as an independent cation channel in the chromaffin granule membrane.

The hydrophobic domain is very likely to be composed of the proton channel (16 kDa) along with subsidiary subunits (120, 40 and 19) whose function is probably its control and regulation. The rest of the complex would be involved in ATP hydrolysis (72 kDa), regulation of ATP hydrolysis (57 kDa) and coupling of both activities (43, 33 and possibly 40 kDa). This distribution of functions within the enzymic complex could not be derived from the results obtained in the present thesis, however, these are perfectly consistent with it and also with results obtained by many other groups that work with similar ATPases (see General Introduction in Chapter 1).

As far as the subunit composition of the chromaffin granule is concerned, ultracentrifugation through glycerol gradients suggests that more subunits than previously thought are associated with the enzymic activities, thus, apart from the five polypeptides traditionally accepted as subunits (120, 72, 57, 40 and 16 kDa), three more (43, 33 and 19 kDa) copurify with the former. This would suggest that the chromaffin granule ATPase is more similar to its counterparts from other animal sources (clathrin-coated vesicles, kidney microsomes) than previously thought.

The experiments performed did not allow us to obtain conclusive evidence about the stoichiometry of the enzymic complex, thus, although rough estimations of the stoichiometry by densitometric scanning of silver-stained gels were consistent with values found in the literature for similar enzymes, these results could not be confirmed by aminoacid analysis. The latter were probably hampered by the low amount of highly pure protein that could be obtained. As a consequence, the less-abundant aminoacids could not be analysed properly and errors due to lipid content in the samples and glycine content in the buffers may have biased the estimated aminoacid composition and transfer efficiency.

The stoichiometry calculated from aminoacid analysis, but not from gel-scanning, suggested the occurrence of 2 subunits of the 57 kDa subunit per 72 kDa subunit. This result is not consistent with the stoichiometry reportedly found for the  $F_1$  domain of F-type ATPases (see Chapter 1) or for the clathrin-coated vesicle V-type ATPase (Arai *et al.*, 1988) although it is interestingly related with the values for substrate and inhibitor binding sites suggested to explain the inhibitory properties of the enzyme. However, it is commonly accepted (see Introduction in Chapter 1) that the 72 kDa subunit is catalytic and the 57 kDa subunit



regulatory which means that the molar ratio between these two subunits should be the opposite to that suggested above. On the other hand, it has also been proposed that the nucleotide binding sites may be located in the interfaces between subunits (Stone *et al.*, 1990) and this model is not in contradiction with our suggestions. Furthermore, a subunit or protomer in the allosteric model is only a "functional" structure that is repeated within an enzyme but does not have to coincide with a given polypeptide that forms part of that particular enzyme and moreover, it is also possible that, under the conditions in which kinetic studies were performed only a limited number of the total binding sites of the enzyme could have been operating, therefore, the experiments may have been showing only part of the complete kinetic behaviour of the enzyme. In any case, it can be concluded that the idea commonly accepted that each of the 72 and 57 kDa subunits have one nucleotide binding site is probably too simple and alternative views must not be discarded.

However, it is worthwhile pointing out again that the information obtained is limited and a proper discussion of the relationship between kinetic and structural properties of the chromaffin granule H<sup>+</sup>-ATPase is not possible at this moment, much work remains to be done in order to obtain conclusive evidence that may allow the characterization of vacuolar ATPases.

The work carried out was designed to make a contribution to the knowledge of vacuolar ATPases. The information obtained during the project is consistent with the general characteristics nowadays accepted for this important group of proton pumps: they are multisubunit enzymes related to F-type ATPases with which they share a basic structure and a distribution of functions amongst the different subunits.

## **7.2. PERSPECTIVES.**

In the last few years great amount of work has been carried out on vacuolar ATPases and, as a consequence, we now have general view of the functional and structural characteristics of these enzymes. However, much remains to be solved and, moreover, further problems have arisen from the studies already performed:

- 1- The composition of vacuolar ATPases is not known accurately and little has been done to define the subunits crucial for the enzymic activities, furthermore, although we have an idea of which subunits are involved in ATP hydrolysis, ATP hydrolysis regulation and proton movement, very little is known about the function and mechanism of action of other subunits of vacuolar ATPases.
- 2- The molecular basis of how proteins can transform scalar chemical energy into substrate movement (vectorial energy) remains obscure and, as in the case of F- and P-type ATPases, the nature of the linkage between ATP hydrolysis and H<sup>+</sup>-translocation in V-type ATPases is unknown .

3- Nothing is known about the relationship between the structure of the subunits and their function, that is, the mechanisms of action at molecular level are not understood.

4- Most experiments have concentrated in the structure of the enzyme and virtually no kinetic studies have been published.

In order to solve these problems several lines of work can be suggested: (1) careful binding studies may provide excellent information about the mechanisms of action of these enzymes and its structural implications; (2) molecular-biological techniques such as site-directed mutagenesis are also needed in order to elucidate subunit structures at the molecular level and to study of the relationship between structure and function of individual subunits. (3) Physical techniques might usefully be applied to the enzyme, to try to demonstrate the postulated ligand-induced conformation change and to measure the value of  $L$ .

Many groups are already cloning and sequencing the various polypeptides supposed to be subunits of vacuolar ATPases and some authors have even been able to obtain yeast strains that do not encode certain subunits of their vacuolar ATPase (Foury, 1990). This all means that research is already under way in order to solve the molecular basis of enzyme catalysis and solute transport not only in vacuolar ATPases but also in many other biological systems.

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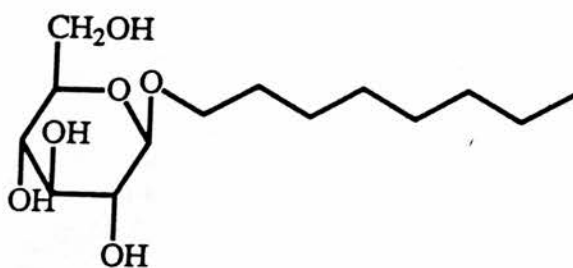
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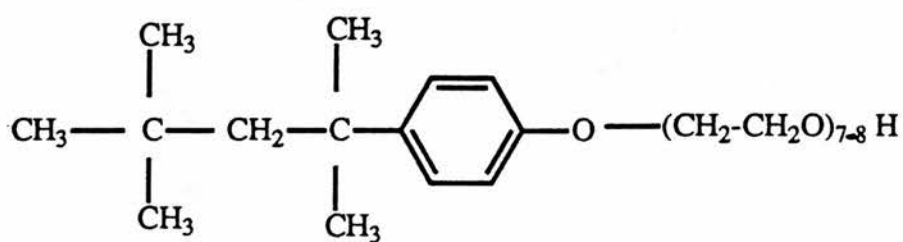
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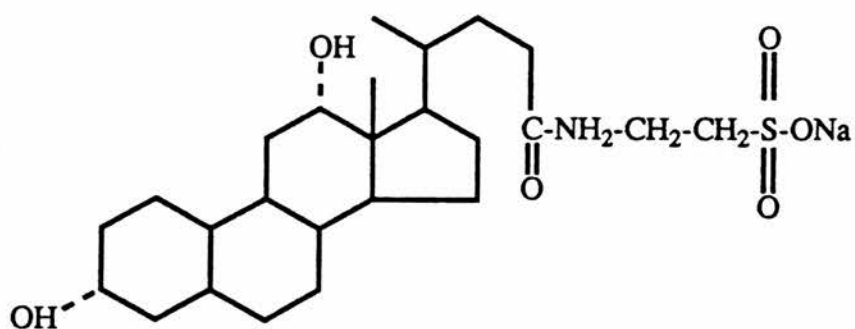
**Appendix 1**  
**CHEMICAL FORMULAE OF SOME COMPOUNDS USED**  
**IN THE PRESENT THESIS**



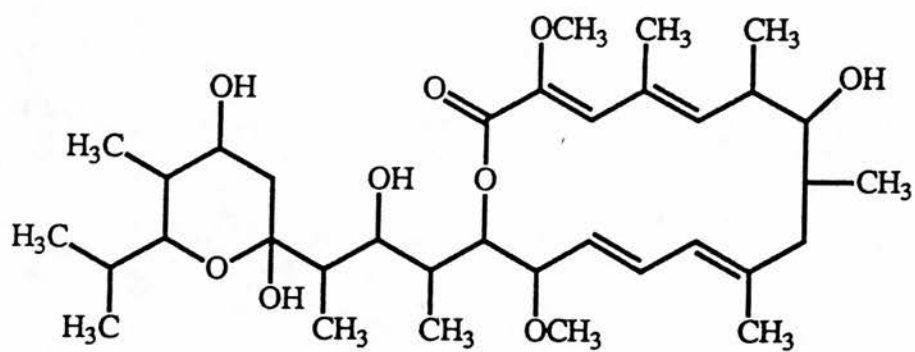
**n-octyl- $\beta$ -glucoside or n-octyl- $\beta$ -D-glucopyranoside**



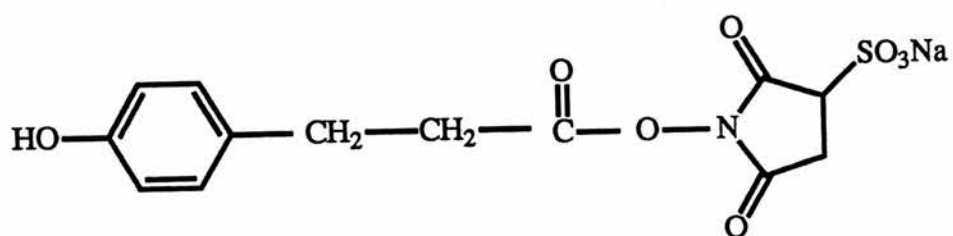
**Triton X-114**



**Sodium taurodeoxycholate**



**Bafilomycin A<sub>1</sub>**



**Sulfosuccinimidyl-3-(4-hydroxyphenyl)propionate**

## **Appendix 2**

### **DERIVATION OF AN EXPRESSION RELATING THE RATE OF ACMA QUENCHING TO THE RATE OF $H^+$ -TRANSLOCATION**

Considering the buffer inside the vesicle:



$$K_a = \frac{[H^+][B^-]}{[HB]}$$

Hence:

$$[HB] = [H^+][B^-]/K_a$$

$$[B^-] = K_a [HB]/[H^+]$$

$$B_t = [HB] + [B^-] = [HB] (1 + (K_a/[H^+]))$$

$$[HB] = \frac{B_t}{1 + (K_a/[H^+])}$$

Similarly:

$$[B^-] = \frac{B_t}{1 + ([H^+]/K_a)}$$

At  $t = 0$ :

$$[H^+]_{in} = K_a \frac{[HB]}{[B^-]}$$

If we assume that:

$$\text{rate of } [H^+] \text{ import} = nkp$$

After a time  $t$ :

$$[H^+]_{in} = K_a \frac{[HB] + nkpt}{[B^-] - nkpt}$$

Substituting:

$$[H^+]_{in} = K_a \frac{\frac{pv[B]}{1 + (K_a/[H^+]_o)} + nkpt}{\frac{pv[B]}{1 + ([H^+]_o/K_a)} - nkpt}$$



Where:

p: vesicular protein concentration (mg/ml)

v: intravesicular volume (ml/mg)

B: intravesicular buffer concentration ( $\mu\text{mol/ml}$ )

$[\text{H}^+]_{\text{in}}$ : internal proton concentration

$[\text{H}^+]_{\text{o}}$ : external proton concentration equal to initial internal proton concentration

$K_a$ : acid dissociation constant of buffer ( $\mu\text{mol/ml}$ )

n:  $\text{H}^+/\text{ATP}$  stoichiometry

k: ATPase specific activity ( $\mu\text{mol/mg/min}$ )

t: time

As shown by Schuldiner *et al.* (1972), fluorescence quenching for weak bases is related to the pH according to the expression:

$$\frac{[\text{H}^+]_{\text{in}}}{[\text{H}^+]_{\text{o}}} = \left( \frac{F_0}{F} - 1 \right) (1/pv)$$

Hence

$$F = \frac{F_0}{1 + (pv[\text{H}^+]_{\text{in}}/[\text{H}^+]_{\text{o}})}$$

$$[\text{H}^+]_{\text{in}} = \frac{K_a (C + Dt)}{E - Dt}$$

Where:

$$C = \frac{pv[B]}{1 + (K_a/[\text{H}^+]_{\text{o}})}$$

$$E = \frac{pv[B]}{1 + ([\text{H}^+]_{\text{o}}/K_a)}$$

$$D = nkp$$

The variation of fluorescence with time would be:

$$\frac{dF}{dt} = \frac{-F_0}{(1 + (pv[\text{H}^+]_{\text{in}}/[\text{H}^+]_{\text{o}}))^2} \cdot \frac{pv}{[\text{H}^+]_{\text{o}}} \cdot \frac{dH}{dt}$$

And:

$$\frac{dH}{dt} = \frac{K_a D}{E - Dt} - \frac{K_a (C + Dt)(-D)}{(E - Dt)^2} = \frac{K_a D (E + C)}{(E - Dt)^2}$$

Hence:

$$\frac{dF}{dt} = \frac{-F_0}{\left[ 1 + \frac{pv}{[\text{H}^+]_{\text{o}}} \frac{K_a (C + Dt)}{(E - Dt)} \right]^2} \cdot \frac{pv}{[\text{H}^+]_{\text{o}}} \cdot \frac{K_a D (E + C)}{(E - Dt)^2}$$

Substituting C, D, and E for their respective values -see above- and re-arranging:

$$\frac{dF}{dt} = \frac{-F_0 v^2 K_a nkp [B]}{[H^+]_0 \left[ \frac{v[B]}{1 + ([H^+]_0/K_a)} (1 - pv) + nkt(pv(K_a/[H^+]_0) - 1) \right]^2}$$

At  $t=0$  this simplifies to:

$$\frac{dF}{dt} = \frac{-F_0 K_a nkp (1 + ([H^+]_0/K_a))^2}{[H^+]_0 [B] (1 - pv)^2}$$

i.e.,  $dF/dt$  is proportional to the protein concentration when  $p$  is small (see Chapter 4) and to the enzyme activity when protein concentration remains constant (kinetic studies).

NOTE: Leakage of protons from the vesicles to the external medium has been considered to be negligible at the beginning of the reaction.

**Appendix 3**  
**PUBLISHED PAPERS**

# Topography of a vacuolar-type H<sup>+</sup>-translocating ATPase: chromaffin-granule membrane ATPase I

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Proteins exposed on the cytoplasmic face of isolated chromaffin granules were labelled by lactoperoxidase-catalysed radioiodination and by non-enzymic biotinylation. Granule membranes were then prepared, and the H<sup>+</sup>-translocating ATPase isolated by fractionation with Triton X-114. The labelling of individual ATPase subunits was assessed by polyacrylamide-gel electrophoresis, followed by autoradiography or by blotting and decoration with <sup>125</sup>I-labelled streptavidin. Subunits of 72, 57 and 40 kDa were strongly labelled, and could be removed from the membrane at pH 11: they are therefore extrinsic proteins. The 120 kDa subunit was also labelled, but it was not solubilized at pH 11. Photolabelling with a hydrophobic probe indicated that this subunit penetrates the bilayer, and enzymic degradation studies showed the presence of N-linked oligosaccharides; this subunit therefore spans the chromaffin-granule membrane. Labelling of the 17 kDa subunit occurred predominantly on the extracytoplasmic (matrix) face of the granule membrane. These results are consistent with this V-type ATPase having a structure that is generally similar to that of mitochondrial (F-type) ATPases, although the attachment of the 120 kDa subunit may be asymmetrical.

## INTRODUCTION

The secretory granules of the adrenal medulla maintain a low internal pH through the action of an ATP-dependent proton pump of the vacuolar type (Al-Awqati, 1986). This enzyme is termed ATPase I, to distinguish it from ATPase II (Apps *et al.*, 1983), a vanadate-sensitive ATPase, apparently of the P type, but which is of unknown function (Moriyama & Nelson, 1988). ATPase I is a multi-subunit complex of molecular mass about 500 kDa; it has been reported to contain five polypeptides of about 115, 70–72, 57, 39–41 and 16–17 kDa (Percy *et al.*, 1985; Cidon & Nelson, 1986), although their stoichiometry has not yet been determined. These polypeptides are referred to as subunits I–V throughout this paper, according to Moriyama & Nelson (1987). Another polypeptide, of 33 kDa, is found in the partially purified ATPase (Percy *et al.*, 1985), but can be removed without loss of ATPase and H<sup>+</sup>-pumping activity (Moriyama & Nelson, 1987).

Subunit II (about 72 kDa) contains the catalytic site (Percy & Apps, 1986; Moriyama & Nelson, 1987), being uniquely labelled by low concentrations of N-ethylmaleimide and protected by ATP. The 17 kDa subunit V is the site of reaction of the inhibitor N,N'-dicyclohexylcarbodi-imide (DCCD) (Sutton & Apps, 1981) and is homologous with the DCCD-reactive subunits of F-type ATPases (Mandel *et al.*, 1988), and so is presumed to form a transmembrane H<sup>+</sup>-conducting channel.

The functions of the other subunits have not yet been defined, and it is not even certain that all are genuine components of the ATPase. Reports of immunological cross-reactivity (Manolson *et al.*, 1986) and, more recently, of sequence similarity between H<sup>+</sup>-translocating

ATPases of the V and F type (Bowman *et al.*, 1988a,b; Zimniak *et al.*, 1988) suggest that subunits II and III (72 kDa and 57 kDa) are related to the  $\alpha$ - and  $\beta$ -subunits of F-type ATPases, and therefore that the overall structures of V- and F-type ATPases may be similar. We now report the use of chemical labelling and enzymic degradation to map the membrane topography of the H<sup>+</sup>-ATPase subunits in intact chromaffin granules. In the chemical-labelling studies, we used lactoperoxidase-catalysed radioiodination and non-enzymic biotinylation to label proteins exposed on the cytoplasmic surface of intact chromaffin granules.

N-Hydroxysuccinimidylbiotin has been used to study the topography of proteins in the plasma membranes of leucocytes by Hurley *et al.* (1985), who concluded that the reagent did not penetrate and therefore only biotinylated those proteins exposed on the extracytoplasmic face of the membrane. We used the more hydrophilic sulphonated derivative (3-sulpho)-N-hydroxysuccinimidyl (SNHS)-biotin, which is even less likely to penetrate the bilayer (Ingalls *et al.*, 1986). Biotinylation has two major advantages: since lysine residues are modified, it is of more general application than the radioiodination of exposed tyrosines, and the detection of biotinylated proteins, with <sup>125</sup>I- or enzyme-conjugated streptavidin, is extremely sensitive. However, biotinylation alters the isoelectric points of proteins, so that two-dimensional gel electrophoresis is not a suitable means of analysis.

<sup>125</sup>I-labelled 3-(trifluoromethyl)-3-(m-[<sup>125</sup>I]iodophenyl)-diazirine (TID) was introduced as a hydrophobic probe for labelling the regions of membrane proteins in contact with the bilayer by Brunner & Semenza (1982). We have used this reagent to label the membrane-embedded polypeptides of ATPase I and have used enzymic

Abbreviations used: SNHS-biotin, (3-sulpho)-N-hydroxysuccinimidylbiotin; [<sup>125</sup>I]TID, 3-(trifluoromethyl)-3-(m-[<sup>125</sup>I]iodophenyl)diazirine; DCCD, N,N'-dicyclohexylcarbodi-imide.

degradation of oligosaccharide chains to investigate which subunits are glycosylated, since glycosylation also has implications for the arrangement of polypeptides in the chromaffin-granule membrane.

## MATERIALS AND METHODS

Crude chromaffin granules were prepared by differential centrifugation of fresh homogenates of bovine adrenal medullae in iso-osmotic (0.3 M) sucrose (Phillips, 1974). Labelling, either with  $^{125}\text{I}^-$  and lactoperoxidase or with SNHS-biotin (described below), was performed on these crude granules, suspended in slightly hyper-osmotic medium, to minimize granule lysis (Hiram *et al.*, 1982). The labelled granules were then further purified by layering 1.0 ml aliquots over 10 ml of 1.7 M-sucrose, 10 mM-Hepes/NaOH, pH 7.0, and centrifugation for 60 min at 50000 rev./min in a Beckman Ti50 rotor (170000 g) at 2 °C. The pelleted granules were lysed by resuspension in 10 ml of 10 mM-Hepes/NaOH, pH 7.0 (0 °C), and the membranes recovered by re-centrifugation.

Radioiodination was performed in a medium of the following composition: crude chromaffin granules, approx. 1 mg of protein/ml; 0.4 M-sucrose; 20 mM-Hepes/NaOH, pH 7.0; 3.7 MBq of  $\text{Na}^{125}\text{I}/\text{ml}$ ; 1.1 unit of glucose oxidase/ml (EC 1.1.3.4; Sigma type IV); 5 mM-glucose; 1.1 units of lactoperoxidase/ml (EC 1.11.1.7; Boehringer); 2  $\mu\text{g}$  of butylated hydroxytoluene/ml; 1  $\mu\text{M}$ -*p*-trifluoromethoxyphenylhydrazine. After incubation for 30 min at approx. 25 °C, the mixture was chilled and approx. 10 mg of chromaffin granules/ml added. The granules were then further purified as described above, and the membranes isolated; during this isolation stage, all solutions contained 1 mM-NaI.

Biotinylation was performed in a medium of the following composition: crude chromaffin granules, 5 mg/ml; 0.35 M-sucrose; 50 mM-Bicine/NaOH, pH 8.3; 0.1 mM-SNHS-biotin (Pierce, Rockford, IL, U.S.A.). After incubation for 30 min at 30 °C, the reaction was stopped by addition of 2 mM-ethanolamine; the granules were then further purified and their membranes isolated; all solutions contained 0.2 mM-ethanolamine.

Trypsin treatment was performed in a medium of the following composition: crude chromaffin granules, 5 mg/ml; 0.35 M-sucrose; 20 mM-Hepes/NaOH, pH 7.0; 10  $\mu\text{g}$  of trypsin/ml (EC 3.4.21.4; Sigma type III). After 60 min at 30 °C, proteolysis was stopped by adding soybean trypsin inhibitor (0.25 mg/ml). The granules were then pelleted, resuspended, biotinylated and purified as described above, and membranes prepared.

To remove extrinsic proteins, chromaffin-granule membranes were suspended in 0.1 M- $\text{Na}_2\text{CO}_3/\text{NaOH}/10$  mM-EDTA, pH 11.0 (Fujiki *et al.*, 1982). After 30 min at 0 °C, the membranes were collected by centrifugation. The washings were dialysed against 1 mM-EDTA, pH 7.0, and freeze-dried.

The 17 kDa DCCD-reactive subunit of the ATPase was extracted from purified chromaffin-granule membranes as described by Sutton & Apps (1981). The chloroform/methanol extract was concentrated by rotary evaporation, and analysed without further purification.

Fractionation of chromaffin-granule membranes with Triton X-114 was performed as described by Pryde & Phillips (1986), giving a detergent-insoluble fraction (P-1), a detergent-rich fraction (P-2) and an aqueous fraction

(S-2). ATPase I was solubilized and further purified from the P-1 fraction, and reconstituted using endogenous lipids (J. R. Perez-Castineira, unpublished work). One- and two-dimensional electrophoretic separation of proteins was performed as described elsewhere (Gavine *et al.*, 1984). After electrophoretic transfer to nitrocellulose sheets (0.45  $\mu\text{m}$  pore size, from Schleicher & Schüll, Dassel, Germany), biotinylated proteins were detected by washing the sheets with the following solutions, all in 150 mM-NaCl/10 mM-Tris/HCl, pH 7.4, at room temperature with gentle shaking: (i) 0.25% (w/v) Tween 20, for 60 min; (ii)  $^{125}\text{I}$ -labelled streptavidin (10 Bq/ $\mu\text{l}$ ; approx. 25 MBq/mg) for 60 min; (iii) 0.05% (w/v) Tween 20 (three changes, 15 min each). For 14 cm  $\times$  16 cm sheets, 50 ml of each solution was used. Sheets were air-dried, then autoradiographed on Hyperfilm MP (Amersham) for 4–24 h at 70 °C, using Kodak intensifying screens. Autoradiographs were scanned with a Joyce-Loebl Chromoscan 3. Streptavidin was obtained from Sigma, and radioiodinated by the Iodogen method (Pierce, Rockford, IL, U.S.A.). Free  $^{125}\text{I}^-$  was removed by passage of the radioiodinated protein (0.2 ml) through a Bio-Gel P6-DG column (1.0 ml) equilibrated with 0.15 M-NaCl, 0.05 M-sodium phosphate; bovine serum albumin (5 mg/ml) was added and the  $^{125}\text{I}$ -labelled streptavidin stored at –10 °C.

Peanut lectin (Sigma) was dissolved at 1 mg/ml in 0.1 M-NaCl/0.05 M-Bicine/NaOH, pH 8.3, and biotinylated by reaction with SNHS-biotin (0.1 mM) for 30 min at 30 °C. After passage through a column of Bio-Gel P6-DG equilibrated with 0.15 M-NaCl, 0.01 M-Tris/HCl, pH 7.4, the lectin was used to decorate blots at a concentration of 10  $\mu\text{g}/\text{ml}$  in this buffer. The blot was then washed and decorated with  $^{125}\text{I}$ -labelled streptavidin, as described above.

For degradation of oligosaccharide chains with neuraminidase, chromaffin-granule membranes (1 mg/ml) or the P-1 fraction from Triton X-114 fractionation (0.25 mg/ml) were incubated for 3 h at 30 °C in 0.2 M-Mes/NaOH buffer, pH 5.5, containing 1.6 mM-benzamidine, 6  $\mu\text{g}$  of pepstatin/ml, 6  $\mu\text{g}$  of leupeptin/ml, 0.4 mM-phenylmethanesulphonyl fluoride, 0.05 mM-*N*- $\alpha$ -*p*-tosyl-L-lysylchloromethane, 0.5 mM-*N*-tosyl-L-phenylalanylchloromethane, and 1 unit of neuraminidase/ml (EC 3.2.1.18, Sigma type VI). For treatment with glycopeptidase F, the conditions were 4 h at 30 °C in 20 mM-Hepes/NaOH, pH 7.4, containing 9 mM-EDTA, 1% (v/v) 2-mercaptoethanol, 6  $\mu\text{g}$  of pepstatin/ml, 6  $\mu\text{g}$  of leupeptin/ml, 1.6 mM-benzamidine and 33 units of glycopeptidase F/ml (EC 3.2.2.18; Boehringer). In some experiments octyl  $\beta$ -glucoside [1% (w/v) final concentration] or SDS (0.1%, w/v) was included.

$^{125}\text{I}$ -labelled TID, of specific activity > 370 TBq/mol, was obtained from Amersham. Chromaffin-granule membranes (0.25 mg) or 0.2 mg of purified reconstituted ATPase I, were incubated with 5 MBq of  $^{125}\text{I}$ -labelled TID (total vol. 0.5 ml) for 15 min in the dark at room temperature, then illuminated for 20 min at 330 nm, in a 5 mm  $\times$  5 mm quartz cuvette in the sample holder of a Perkin-Elmer fluorimeter. Labelled proteins were analysed by electrophoresis and autoradiography.

Silver staining of gels was performed according to Wray (1981). Apparent molecular masses of protein bands were calculated from the mobilities of the following standard proteins (kDa):  $\beta$ -galactosidase (116),



phosphorylase *b* (97), serum albumin (66.25), ovalbumin (45), carbonic anhydrase (30), trypsin inhibitor (20.1) and lysozyme (14.3).

## RESULTS

Fractionation of chromaffin-granule membranes by phase-separation in Triton X-114 (Pryde & Phillips, 1986) is a rapid and convenient procedure that yields three fractions, termed P-1, P-2 and S-2. In P-1, ATPase I is purified at least 10-fold, having a specific activity of about  $2 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  when resolubilized (J. R. Perez-Castineira, unpublished work); its subunits can be recognized in SDS/polyacrylamide gels by comparison with the purified enzyme (Percy *et al.*, 1985) and the only major contaminants are proteins of apparent molecular mass 33 and 36 kDa. Fraction P-2 contains the membrane proteins dopamine  $\beta$ -hydroxylase, cytochrome  $b_{561}$  and ATPase II, and S-2 contains extrinsic proteins, notably chromogranins and the soluble form of dopamine  $\beta$ -hydroxylase. Triton X-114 fractionation, followed by one- or two-dimensional polyacrylamide-gel electrophoresis, provided a satisfactory method for the rapid analysis of ATPase I subunits, after labelling of intact chromaffin granules.

### Labelling of lactoperoxidase-catalysed radioiodination

Crude, intact chromaffin granules were subjected to radioiodination, then re-purified to remove contaminating organelles and membrane fragments; membranes were then isolated from these granules, fractionated and analysed electrophoretically. A one-dimensional electrophoretogram is shown in Fig. 1. In the Triton-insoluble fraction P-1 (track 2), ATPase I subunits II (72 kDa), III (57 kDa) and IV (40 kDa) are strongly labelled, and subunit I (122 kDa) is weakly labelled. Subunit V (17 kDa) is unlabelled; the smallest labelled polypeptide, of apparent molecular mass 15 kDa, is not a component

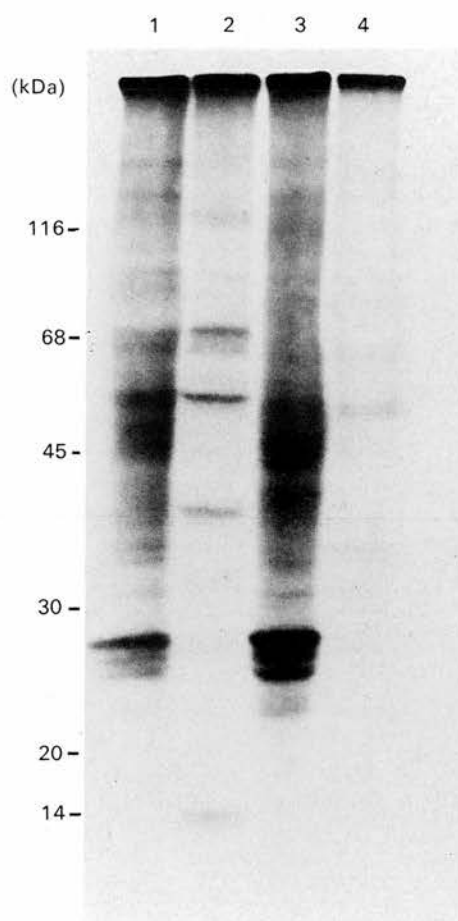


Fig. 1. Triton X-114 fractionation of membranes from radiolabelled chromaffin granules: autoradiograph

Track 1, chromaffin-granule membranes (100  $\mu\text{g}$ ); track 2, P-1 fraction (60  $\mu\text{g}$ ); track 3, P-2 fraction (60  $\mu\text{g}$ ); track 4, S-2 fraction (50  $\mu\text{g}$ ).

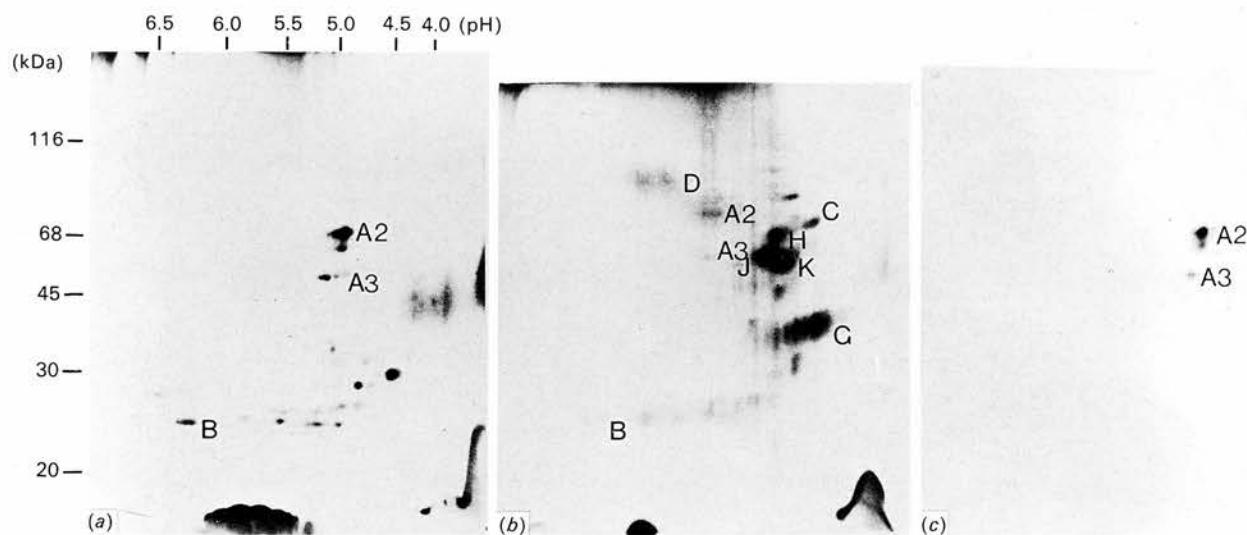


Fig. 2. Two-dimensional electrophoretic separation of radioiodinated chromaffin-granule membrane proteins: autoradiographs

(a) Membranes from radioiodinated granules (150  $\mu\text{g}$ ); (b) membranes radioiodinated after isolation (150  $\mu\text{g}$ ); (c) proteins solubilized at pH 11, after radioiodination of intact granules (50  $\mu\text{g}$ ). Abbreviations used; A2, ATPase I subunit II; A3, ATPase I subunit III; B, cytochrome  $b_{561}$ ; C, chromogranin A; D, dopamine  $\beta$ -hydroxylase; G, glycoprotein III; H, glycoprotein H; J, glycoprotein J; K, glycoprotein K.

of ATPase I. The integrity of the granules is confirmed by the labelling patterns shown in fractions P-2 (track 3) and S-2 (track 4), which provide a control for this experiment. Cytochrome  $b_{561}$  is a 30-kDa transmembrane protein (Duong & Fleming, 1984; Perin *et al.*, 1988), although its electrophoretic mobility suggests a lower molecular mass. It is strongly iodinated (track 3), whereas dopamine  $\beta$ -hydroxylase and the chromogranins, located in the granule matrix (70–75 kDa), are not iodinated (tracks 3 and 4). No labelling was detected in the soluble proteins released on lysis of the granules (not shown).

Two-dimensional electrophoresis was also used to separate radioiodinated membrane proteins. The position of subunit II (72 kDa) in these gels has previously been demonstrated by [ $^3\text{H}$ ]N-ethylmaleimide labelling (Percy

& Apps, 1986); both it and subunit III (57 kDa) are iodinated (Fig. 2a), but no other subunits of ATPase I are visible on this autoradiograph, probably because they fail to enter the first-dimension (electrofocusing) gel. If chromaffin-granule membranes, as opposed to intact granules, are radiolabelled, many additional proteins are labelled (Fig. 2b); these include dopamine  $\beta$ -hydroxylase, glycoprotein III and the glycoproteins previously termed H, J and K (Gavine *et al.*, 1984; Wood *et al.*, 1985), two of which were subsequently identified as isoforms of the carboxypeptidase involved in neuropeptide processing (Laslop *et al.*, 1986). Fig. 2(c) shows the radioiodinated polypeptides that could be removed from the membranes by washing at high pH; these include subunits II and III of ATPase I.

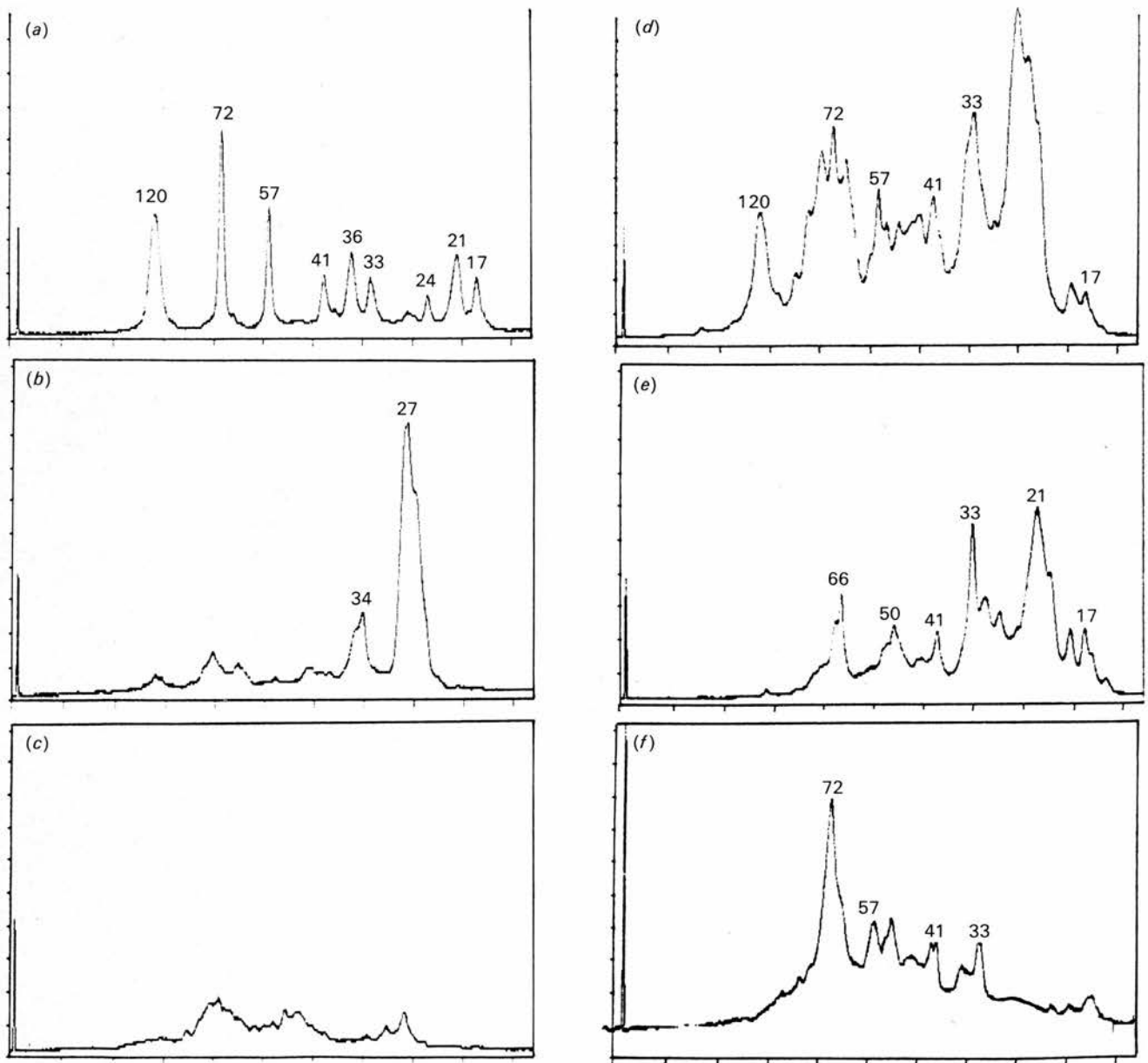


Fig. 3. Biotinylation of chromaffin-granule membrane proteins: densitometric scans of autoradiographs

(a) P-1 fraction (60  $\mu\text{g}$ ); (b) P-2 fraction (50  $\mu\text{g}$ ); (c) S-2 fraction (76  $\mu\text{g}$ ); (d) unfractionated membranes (130  $\mu\text{g}$ ); (e) unfractionated membranes from trypsin-treated granules (100  $\mu\text{g}$ ); (f) proteins solubilized at pH 11 (50  $\mu\text{g}$ ). Full scale, absorbance = 2.0. Numbers above peaks are apparent molecular masses (kDa).



### Labelling by biotinylation

Chromaffin granules were labelled with SNHS-biotin at pH 8.3 and 30 °C, in slightly hyper-osmotic sucrose. Granules were then re-purified, and membranes prepared and fractionated with Triton X-114. This technique was also applied to trypsin-treated granules. Fig. 3 presents densitometric scans of autoradiograms obtained from individual gel tracks; the densities are all to the same absorbance scale. Electrophoretic migration was from left to right and the apparent molecular masses of individual peaks are indicated. All of the subunits of ATPase I are biotinylated and appear in the P-1 fraction (Fig. 3a). As expected in intact granules, cytochrome  $b_{561}$  is labelled, and dopamine  $\beta$ -hydroxylase is not (Fig 3b); there is very little labelling of proteins in the S-2 fraction (Fig. 3c), which consists mainly of soluble proteins from the granule matrix. As with lactoperoxidase-catalysed radioiodination, no labelling was detected in the soluble proteins released on lysis of the granules (not shown).

Subunits I, II, III and IV are major biotinylated components of unfractionated chromaffin-granule membranes (Fig. 3d); trypsin treatment of the granules degrades subunit II from 72 to 66 kDa, and subunit III from 57 to 50 kDa (Fig. 3e). The molecular masses of the degradation products of subunits II and III were established by Triton X-114 fractionation of membranes from trypsin-treated granules (not shown). Subunit I is more extensively proteolysed and cannot be recognized in Fig. 3(e), nor was it found after Triton X-114 fractionation. As reported elsewhere (Apps *et al.*, 1984), cytochrome  $b_{561}$  is clipped to an apparent molecular mass of 21 kDa, during proteolysis of chromaffin granules. Washing the membranes at pH 11 elutes subunits II, III and IV, but not I or V (Fig. 3f), nor cytochrome  $b_{561}$ , known to be an intrinsic membrane protein.

The results of these experiments can be summarized as follows: (1) all five subunits of the  $H^+$ -ATPase are accessible to SNHS-biotin in intact granules and must therefore be exposed on the cytoplasmic face of the granule membrane; (2) washing at pH 11 solubilizes subunits II, III and IV, but not I or V, which must therefore be intrinsic membrane proteins; (3) trypsin treatment of intact granules leads to extensive degradation of subunit I, and partial degradation of subunits II and III, confirming that all three are exposed on the cytoplasmic face of the granule membrane.

### Extraction of subunit V

Although subunit V is biotinylated in intact granules (Fig. 3a; 17 kDa), its labelling is weak in comparison with that of other polypeptides and in relation to the intensity of protein staining. This hydrophobic polypeptide is soluble in chloroform/methanol (Sutton & Apps, 1981) and was extracted from membranes prepared from biotinylated granules, and from membranes which had been biotinylated after isolation. Extracts from equal quantities of membranes were then run on a 10–15% (w/v) polyacrylamide gel and their biotinylation compared (Fig. 4). Labelling of the 17 kDa polypeptide was far more intense in membranes than in intact granules (note the difference in scales).

### Enzymic deglycosylation

Purified chromaffin-granule membranes were treated

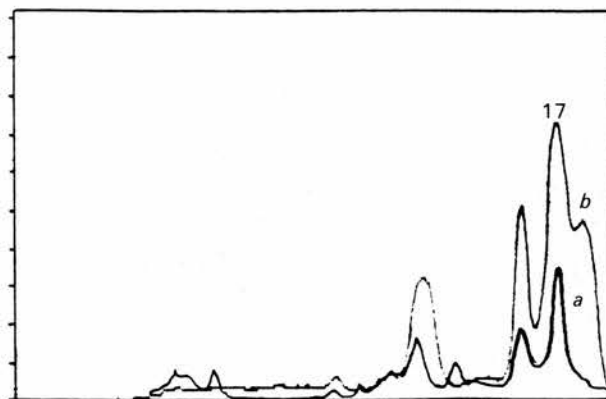


Fig. 4. Biotinylation of subunit V: densitometric scan of autoradiograph

Chloroform/methanol extracts of chromaffin-granule membranes (a) prepared from biotinylated granules (2.2  $\mu$ g of protein; full scale, absorbance = 0.5) and (b) biotinylated after isolation (3.4  $\mu$ g of protein; full scale, absorbance = 2.0).

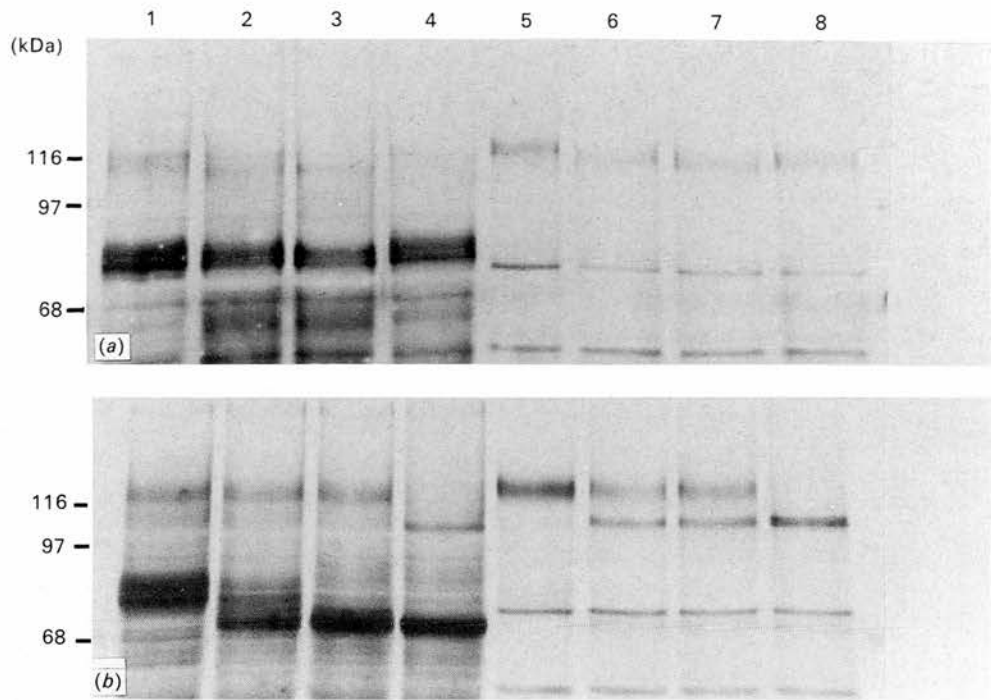
with neuraminidase and with glycopeptidase F, at the appropriate pH values for these enzymes, and in the presence of proteinase inhibitors. Digested membrane proteins were separated on 6–15% (w/v) polyacrylamide gels which were silver-stained. Of the putative ATPase I polypeptides, only subunit I was affected. Neuraminidase treatment produces a slight reduction in its apparent molecular mass (Fig. 5a). As with several other proteins of the chromaffin-granule membrane (Gavine *et al.*, 1984), this polypeptide binds the galactose-specific peanut lectin after neuraminidase treatment, but not before (not shown). However, this lectin binds non-specifically to several other proteins in the P-1 fraction. More convincing evidence for glycosylation of this subunit is the significant reduction in molecular mass (from 120 to 110 kDa) that is produced by digestion with glycopeptidase F (Fig. 5b). No other subunit of ATPase I is affected. The faint band of apparent molecular mass about 71 kDa that appears on glycopeptidase F treatment (Fig. 5b, tracks 6–8) is the deglycosylated form of dopamine  $\beta$ -hydroxylase, present as a contaminant in the P-1 fraction; it is seen more clearly in tracks 3 and 4.

### Labelling with [ $^{125}$ I]TID

In chromaffin-granule membranes, the hydrophobic photoactivated probe  $^{125}$ I-labelled TID labels subunits I and V of the  $H^+$ -ATPase; these are the most intensely labelled of all polypeptides and, as expected, cytochrome  $b_{561}$  is also labelled (Fig. 6a). (Note that in this scan, subunit V is not resolved from other components of low molecular mass, which are probably lipids.) With purified, reconstituted ATPase I, the results are even more clear-cut (Fig. 6b); subunits I and V are the only labelled polypeptides.

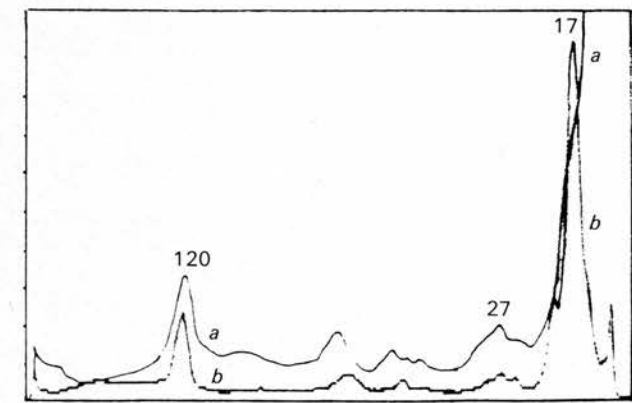
### DISCUSSION

The results for individual ATPase I subunits are summarized in Table 1, which also includes data on the 33 kDa polypeptide that was originally postulated to be a subunit of ATPase I (Percy *et al.*, 1985), but which can



**Fig. 5. Enzymic degradation of oligosaccharide chains: upper parts of silver-stained SDS/polyacrylamide gels**

Tracks 1–4, chromaffin-granule membranes; tracks 5–8, P-1 fraction. (a) Neuraminidase digestion; (b) glycopeptidase-F digestion. Tracks 1 and 5, controls (no enzyme); 2 and 6, digested without detergent; 3 and 7, with 1 % (w/v) octyl  $\beta$ -glucoside; 4 and 8, with 0.1 % (w/v) SDS.



**Fig. 6. Photolabelling with [<sup>125</sup>I]TID: densitometric scan of autoradiograph**

(a) Chromaffin-granule membranes; (b) purified ATPase I. Full scale, absorbance = 1.0.

be removed. A polypeptide of similar molecular mass is found in the ATPase of clathrin-coated vesicles (Arai *et al.*, 1987), but its function in the chromaffin granule is in doubt.

Subunit I is clearly exposed on the cytoplasmic face of the chromaffin-granule membrane and can be radioiodinated, biotinylated or proteolysed in intact granules. It is glycosylated, which suggests that it is a transmembrane protein, since the oligosaccharide chains of chromaffin-granule membrane glycoproteins, like those of other endomembrane glycoproteins, are located exclusively on the extracytoplasmic face of the granule (Huber *et al.*, 1979). In agreement with this, it is not solubilized at pH 11 and is labelled by [<sup>125</sup>I]TID, though in comparison subunit V is more strongly labelled. The polypeptide chain of subunit I may only traverse the bilayer a small number of times or it may be screened by other subunits.

Subunits II, III and IV behave as extrinsic proteins on

**Table 1. Summary of properties of ATPase I subunits**

Abbreviations used: LPO, lactoperoxidase; Glyco-F, glycopeptidase F.

Subunit	Molecular mass (kDa)	Labelling			Degradation		Solubilization at pH 11
		<sup>125</sup> I <sup>-</sup> /LPO	[ <sup>125</sup> I]-TID	SNHS-biotin	Trypsin	Glyco-F	
I	120	+	++	++	++	+	—
II	72	++	—	++	+	—	+
III	57	++	—	++	+	—	+
IV	41	++	—	+	—	—	+
V	17	—	++	(+)	—	—	—

the cytoplasmic face of the membrane, being accessible to non-penetrating reagents, solubilized at pH 11 and not glycosylated. They show no labelling by [ $^{125}$ I]TID, so are not embedded in the bilayer.

Subunit V can be slightly biotinylated in intact granules, but is much more strongly labelled in membrane fragments. Its amino acid sequence has recently been reported (Mandel *et al.*, 1988): its structure was predicted to consist of four transmembrane helices, connected by one cytoplasmic and two intragranular loops, with two lysines exposed on each face of the membrane. The *N*-terminus is blocked (Sutton & Apps, 1981). The strong labelling in membrane fragments suggests that labelling occurs mainly on the extracytoplasmic (matrix) face and that interactions with other subunits probably prevent labelling on the cytoplasmic face. The slight labelling of subunit V in intact granules may even be of damaged ATPase molecules, in which subunits II–IV have been lost, leaving subunit V accessible.

These results are consistent with a model in which subunit V (17 kDa) is intrinsic and subunits II–IV (72, 57 and 41 kDa) form an extrinsic complex on the cytoplasmic face of the granule membrane, protecting subunit V from labelling. This subunit arrangement is like that of  $F_1F_0$ -ATPases, and this general similarity between ATPases of the V- and F-types has been demonstrated by immunological studies (Manolson *et al.*, 1987) and sequence determinations (Bowman *et al.*, 1988a,b; Mandel *et al.*, 1988). It is, however, difficult to reconcile the properties of subunit I (120 kDa) with an  $F_1F_0$ -like structure. Although its polypeptide stoichiometry is unknown, the ATPase (molecular mass about 500 kDa) is unlikely to contain more than one copy of subunit I (120 kDa). This could imply that subunit I is associated asymmetrically with the complex. An alternative possibility, that it functions as a central core, seems unlikely, as no polypeptide larger than subunit II has been found in the V-type ATPases of plants and fungi (Bowman & Bowman, 1986), and the  $H^+$ -ATPase of kidney cortex may also lack this subunit (Gluck & Caldwell, 1987, 1988). However, a component of similar molecular mass and properties occurs in the  $H^+$ -ATPase of clathrin-coated vesicles (Xie & Stone, 1986; Arai *et al.*, 1987). It therefore seems that, if subunit I is really a component of some V-type ATPases, it does not have a fundamental role in catalysis, but may have a regulatory function.

The  $H^+$ -translocating ATPases isolated from kidney microsomes and from clathrin-coated vesicles have reported subunit compositions that are more complex than that of the chromaffin-granule enzyme, but their morphology is similar. For the kidney enzyme, subunits of 70 and 56 kDa (presumably corresponding to subunits II and III) have been located on the cytoplasmic face of the membrane by immunological methods and electron microscopy suggests a 9.5 nm cytoplasmic domain that is morphologically similar to that of F-type ATPases (Brown *et al.*, 1987, 1988). Recent studies on the coated-vesicle ATPase (Arai *et al.*, 1988) have also resulted in a structural model that is in most respects similar to that proposed here for the chromaffin-granule enzyme. The major differences are that the coated-vesicle ATPase may have more subunit types, polypeptides of 38, 34, 33 and 19 kDa being present in addition to polypeptides apparently similar to the subunits I–V described here, and that subunit I (100 kDa in the coated-vesicle ATPase) is

not glycosylated. A subunit of 40 kDa was found to be integral to the membrane, but this may not be equivalent to subunit IV in the chromaffin-granule enzyme. The subunit stoichiometry of the coated-vesicle ATPase was elegantly measured by amino acid analysis (Arai *et al.*, 1988) and a more refined model of the structure of the chromaffin granule enzyme must await similar studies on this ATPase.

#### Note added in proof (received 2 August 1989)

Moriyama & Nelson (1989) have recently reported that subunits of 72, 57 and 34 kDa are released from chromaffin granules during cold-inactivation of ATPase I. Those results are entirely consistent with those obtained in the labelling studies reported here.

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# Vacuolar H<sup>+</sup>-ATPase of adrenal secretory granules

## Rapid partial purification and reconstitution into proteoliposomes

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A procedure has been developed for the rapid purification and reconstitution into phospholipid vesicles of the proton-translocating ATPase of bovine adrenal chromaffin-granule membranes. It involves fractionation of the membranes with Triton X-114, resolubilization of the ATPase with n-octyl glucoside, addition of purified lipids and removal of detergent by gel filtration. The entire process can be completed within 2 h. H<sup>+</sup> translocation was detected by the ATP-dependent quenching of the fluorescence of a permeant weak base. The effect of varying the lipid composition of the vesicles on ATP hydrolysis and H<sup>+</sup> translocation by the reconstituted enzyme was examined. ATPase activity was maximally increased about 4-fold by added lipid, but was relatively insensitive to its composition, whereas vesicle acidification was absolutely dependent on the addition of phospholipids and cholesterol.

## INTRODUCTION

H<sup>+</sup>-translocating ATPases fall into three classes, called F, P and V (Pederson & Carafoli, 1987). Those of the V (vacuolar) type occur in the tonoplasts of higher plants, in fungal vacuolar membranes and in endomembranes bounding the acidic compartments of animal cells (Forgac, 1989). Animal V-type ATPases have been isolated from a number of sources, including secretory granules (Percy *et al.*, 1985; Cidon & Nelson, 1986), clathrin-coated vesicles (Xie & Stone, 1986) and kidney medulla microsomes (Gluck & Caldwell, 1988) and also identified in the Golgi (Young *et al.*, 1988; Moriyama & Nelson, 1989b). They contain at least six (and possibly more) types of subunit (Forgac, 1989) and have a structure that is generally similar to that of F-type ATPases (Arai *et al.*, 1988; Apps *et al.*, 1989; Moriyama & Nelson, 1989a). Immunological cross-reactivity has been demonstrated among V-type ATPases from various sources, the F-type ATPases from eubacteria and the H<sup>+</sup>-ATPases from archaeobacteria (Manolson *et al.*, 1989), and comparison of the amino acid sequences of the two nucleotide-binding subunits confirms this similarity, suggesting that V- and F-type ATPases have a common evolutionary origin (Gogarten *et al.*, 1989; Sudhof *et al.*, 1989).

Published procedures for the isolation of animal V-type ATPases involve several steps and yield a product of specific activity 3–5  $\mu\text{mol/min per mg}$  of protein (Moriyama & Nelson, 1987, 1989a). The fractionation of chromaffin-granule membranes by phase separation in Triton X-114 (Bordier, 1981; Pryde & Phillips, 1986) affords extensive purification of the H<sup>+</sup>-ATPase complex in a single step, but the product is of low specific activity (Percy *et al.*, 1985). In order to investigate the properties of this ATPase we required a reconstituted enzyme capable of H<sup>+</sup> translocation. We now report an adaptation of the Triton X-114 procedure that yields H<sup>+</sup>-pumping vesicles of high specific ATPase activity and can be completed within 2 h.

Chromaffin-granule membranes contain a P-type ATPase of unknown function, termed 'ATPase II' (Percy *et al.*, 1985; Moriyama & Nelson, 1988), as well as the H<sup>+</sup>-translocating V-type ATPase (ATPase I). The former ATPase is separated during the first stage of purification, appearing in the detergent-rich phase of Triton X-114 fractionation (Pryde & Phillips, 1986).

## MATERIALS AND METHODS

### Materials

Triton X-114 was obtained from Fluka AG, Buchs, Switzerland, and purified by the method of Bordier (1981). n-Octyl  $\beta$ -glucoside was purchased from Sigma. Bio-Gel P6-DG was from Bio-Rad. Cholesterol was supplied by Boehringer, Lewes, East Sussex, U.K. Bovine spinal-cord phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine were obtained from Lipid Products, Nutfield, Redhill, Surrey, U.K. Soybean phosphatidylcholine was from Karlshamns AB, Stockholm, Sweden. 9-Amino-6-chloro-2-methoxyacridine (ACMA) and bafilomycin A<sub>1</sub> were generously given by Dr. R. Kraayenhof, Free University, Amsterdam, The Netherlands, and Dr. K. Altendorf, University of Osnabrück, Osnabrück, Germany.

### Analytical methods

ATP hydrolysis was measured at 34 °C (see Figs. 1–6 below) or 37 °C (see Table 1 below), by a coupled spectrophotometric assay described previously (Percy *et al.*, 1985), with the addition of carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) (2  $\mu\text{M}$ ). H<sup>+</sup> translocation by reconstituted ATPase proteoliposomes was measured at 30 °C by recording the quenching of ACMA fluorescence in a Perkin-Elmer 3000 fluorimeter, with excitation and emission wavelengths of 420 and 480 nm respectively. The assay medium (0.5 ml) contained 0.3 M-sucrose, 10 mM-Hepes/NaOH buffer, pH 7.4, 3 mM-ATP, 3 mM-MgSO<sub>4</sub>, ACMA (0.2  $\mu\text{g/ml}$ ), valinomycin (0.4  $\mu\text{g/ml}$ ) and reconstituted ATPase vesicles (4  $\mu\text{g}$  of protein/ml). Protein concentrations were measured by an adaptation of the Folin-Lowry method (Peterson, 1977). Concentrations of cholesterol and of phospholipids were measured by spectrophotometric assays, using assay kits purchased from Boehringer. Triton X-114 was assayed by the method of Garewal (1973). Concentrations of bafilomycin A<sub>1</sub> were determined spectrophotometrically, using the published extinction coefficient of 25  $\text{mm}^{-1} \text{cm}^{-1}$  (Werner *et al.*, 1984).

### Purification of the H<sup>+</sup>-ATPase

Chromaffin-granule membranes were purified from fresh bovine adrenal medullae as described elsewhere (Apps *et al.*,

Abbreviations used: ACMA, 9-amino-6-chloro-2-methoxyacridine; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

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1980) and stored at  $-20^{\circ}\text{C}$  in 10 mM-Hepes/NaOH (pH 7.4)/0.1 mM-EDTA/1 mM-dithiothreitol. After thawing, membranes were sedimented (Beckman TL-100.3 rotor; 100 000 rev./min, 10 min; 412 000  $g_{av.}$ ). The membrane pellet was resuspended in 0.15 M-KCl/10 mM-Tris/HCl (pH 7.4)/1 mM-EDTA/1 mM-dithiothreitol, with gentle homogenization in a Teflon/glass homogenizer. Triton X-114 was added so that the final concentrations were: detergent, 20 mg/ml; membranes, 4 mg of protein/ml. After 5 min at  $0^{\circ}\text{C}$ , the white pellet was removed by centrifugation as described above and washed by homogenization in the same buffer, containing Triton X-114 (20 mg/ml), followed by centrifugation. It was then resuspended in half the original volume of buffer, containing n-octyl  $\beta$ -glucoside (18 mg/ml); after 10 min at  $0^{\circ}\text{C}$  it was centrifuged and the clear supernatant carefully removed. This was the solubilized partially purified ATPase.

### Reconstitution into phospholipid vesicles

Pure lipids [solutions in chloroform/methanol (2:1, v/v)] were dispensed into glass tubes in the required amounts and dried under a stream of  $\text{N}_2$ . (Typically, for 0.2 ml of resolubilized ATPase, 350  $\mu\text{g}$  of phosphatidylcholine, 350  $\mu\text{g}$  of phosphatidylethanolamine, 115  $\mu\text{g}$  of phosphatidylserine and 215  $\mu\text{g}$  of cholesterol gave near-optimal activities; see the Results section.) The solubilized ATPase (0.2 ml) was added and the lipids dissolved by vortex-mixing, followed by gentle homogenization. The solution was incubated at room temperature (about  $20^{\circ}\text{C}$ ) for 10 min and loaded on to a 1 ml column of Bio-Gel P6-DG that had been equilibrated with 0.15 M-KCl/10 mM-Tris/HCl (pH 7.4)/1 mM-dithiothreitol/1 mM-EDTA/10% (w/v) methanol, and packed by centrifugation in a bench centrifuge (1 min; 1400 rev./min, 150  $g_{av.}$ ). The sample was passed through the column by centrifugation under the same conditions, and stored on ice after collection.

## RESULTS

### Purification of $\text{H}^+$ -ATPase

Table 1 summarizes the purification of the  $\text{H}^+$ -ATPase. Although there is a considerable loss of activity in the second step, washing of the pellet is essential to minimize contamination with ATPase II and endogenous lipids. Some ATPase activity is found in the supernatant, but it is partially vanadate-sensitive and therefore represents ATPase II (Percy *et al.*, 1985). The final specific activity reported in Table 1 is that obtained on reconstitution with a mixture of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and cholesterol, in the proportion defined in the Materials and methods section; this is the optimal lipid composition that we have found, but it may be possible to achieve higher activities with other combinations of lipids.

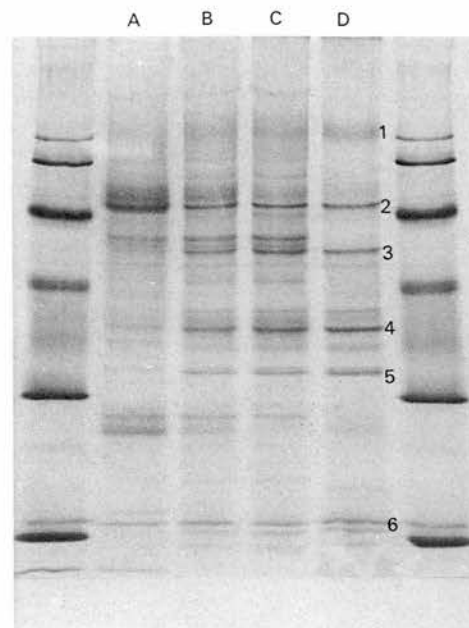


Fig. 1. SDS/PAGE of ATPase fractions

Track A, chromaffin-granule membranes; track B, protein precipitated with Triton X-114; track C, washed precipitate; track D, reconstituted ATPase; outer tracks, molecular-mass standards [from top to bottom:  $\beta$ -galactosidase (116 kDa), phosphorylase *b* (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa)]. Tracks A–D contained 50  $\mu\text{g}$  of protein each. Staining was with Coomassie Blue. The numbers to the right of track D indicate the ATPase subunits (120, 72, 57, 41, 33 and 16 kDa).

Fig. 1 shows an SDS/polyacrylamide gel of the ATPase through the various stages of purification. The reconstituted enzyme contains the six major polypeptides of 120, 72, 57, 41, 33 and 16 kDa that have been assigned to the ATPase (Percy *et al.*, 1985; Moriyama & Nelson, 1987) and contaminants of 78, 38 and 36 kDa. It is noteworthy that reconstitution is selective, the proportion of contaminant proteins decreasing during the last step.

The procedure was usually applied to about 6 ml of purified chromaffin-granule membranes (24 mg of protein); on a smaller scale, mechanical losses became significant and the overall recovery was reduced. The reconstituted ATPase had good stability and lost only 4–10% of its ATPase activity in 24 h at  $4^{\circ}\text{C}$ . However, at the penultimate stage (solution in octyl glucoside) activity was rapidly lost, and it was important to proceed at once with the reconstitution.

Table 1. Reconstitution of chromaffin-granule ATPase 1

The data shown are for a typical preparation; values in parentheses are the means  $\pm$  S.D. of specific activities and recoveries obtained in four separate preparations.

Stage	ATPase activity (nmol/min per ml)	[Protein] (mg/ml)	Specific activity (nmol/min per mg)	Recovery (%)
Membranes	2815	7.6	370 (348 $\pm$ 26)	100
Precipitate	1546	1.4	1104 (1263 $\pm$ 222)	90.0 (87.7 $\pm$ 6.9)
Washed precipitate	711	1.0	711 (1120 $\pm$ 355)	19.7 (26.1 $\pm$ 4.3)
Solubilized ATPase	200	0.21	952 (894 $\pm$ 195)	6.8 (7.0 $\pm$ 1.8)
Reconstituted ATPase	500	0.21	2380 (2453 $\pm$ 139)	15.1 (16.6 $\pm$ 1.9)

In the final step, 0.15 M-KCl could be replaced by 0.3 M-sucrose. The resulting vesicles initially had similar ATPase and H<sup>+</sup>-translocating activity (provided KCl was added to the assay medium; see below), but were less stable than those containing KCl. Similarly, if methanol was omitted from the reconstitution buffer, the resulting vesicles were less active in both assays, and also lost activity rapidly on storage.

Assay of H<sup>+</sup> translocation

The quenching of ACMA fluorescence provides a sensitive and convenient assay for the development of a transmembrane pH difference. A typical trace of fluorescence quenching by reconstituted ATPase vesicles is shown in Fig. 2. Initial rates of fluorescence quenching were directly proportional to the amount of ATPase added, at least up to 4 µg of protein/ml (results not shown) and were absolutely dependent on added ATP and Mg<sup>2+</sup>, and also on valinomycin (see below). Quenching was reversed by the addition of the uncoupler FCCP. ATPase activity and H<sup>+</sup> translocation were inhibited in parallel by bafilomycin A<sub>1</sub> (Bowman *et al.*, 1988), 50 % inhibition occurring at 1 nmol/mg of protein (results not shown).

Conditions for optimal activity

Both the ATPase and the H<sup>+</sup>-translocation activities were dependent on the lipid/protein ratio in the reconstituted vesicles (Fig. 3). These results were obtained with the mixture of lipids that gave optimal activities (see below), but the optimal lipid/

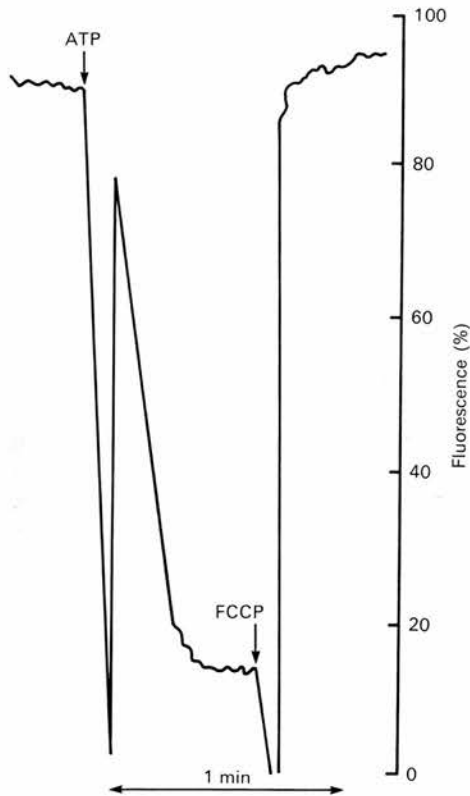


Fig. 2. Typical time course of ACMA fluorescence quenching by reconstituted ATPase vesicles (4 µg of protein/ml)

Additions of ATP (3 mM) and FCCP (4 µM) were made at the points shown. The vesicles contained phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and cholesterol in the proportions 9:9:3:5 (by wt.), and the lipid/protein ratio was 26:1. Valinomycin (0.4 µg/ml) was present throughout.

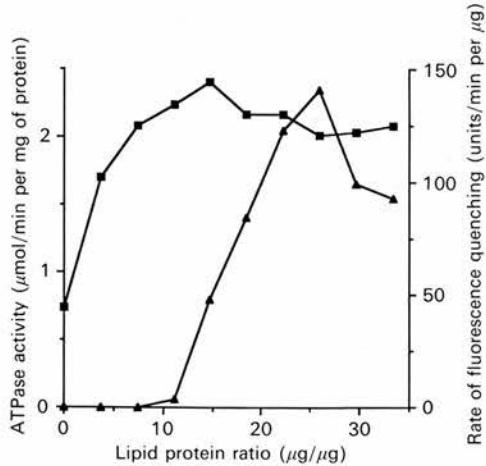


Fig. 3. Dependence of ATPase activity (■) and ACMA fluorescence quenching rate (▲) on the lipid/protein ratio

The lipid composition was the same as in Fig. 2, and valinomycin (0.4 µg/ml) was present.

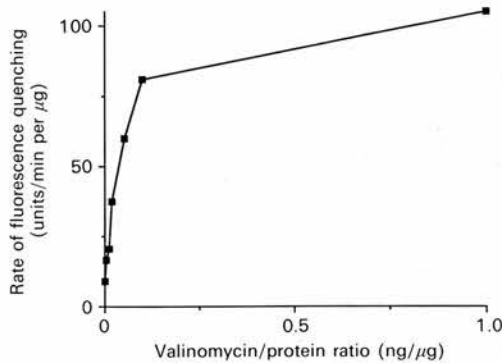


Fig. 4. Dependence of initial rate of quenching of ACMA fluorescence on valinomycin concentration

The vesicle concentration was 4 µg of protein/ml and vesicle composition was the same as in Fig. 2.

protein ratio was not dependent on the lipid composition; with each lipid mixture tried, vesicles containing 26 µg of total lipid/µg of protein had maximal H<sup>+</sup>-translocating activity, and almost maximal ATPase activity. Similar activities were obtained using phospholipids from soybean and from bovine spinal cord.

Since H<sup>+</sup> translocation is electrogenic, the maximal activity is found when the membrane potential induced by H<sup>+</sup> translocation is overcome by compensating ion movements. In these experiments this was achieved by the addition of valinomycin, which permits exit of K<sup>+</sup> from the vesicles in exchange for H<sup>+</sup>. At very low concentrations of valinomycin, H<sup>+</sup> translocation was apparently limited by the rate of K<sup>+</sup> movement (Fig. 4); in all other experiments we used approx. 100 ng of valinomycin/µg of protein. ATPase activity is also stimulated by valinomycin and by FCCP; the stimulation observed depended on the lipid/protein ratio in the vesicles (results not shown), but was up to 50 %. ATPase activity was therefore routinely assayed in the presence of FCCP.

Effect of lipid composition on ATPase and H<sup>+</sup>-translocating activities

In order to examine the effect on the reconstituted ATPase of the lipid composition of the vesicles, we made vesicles containing



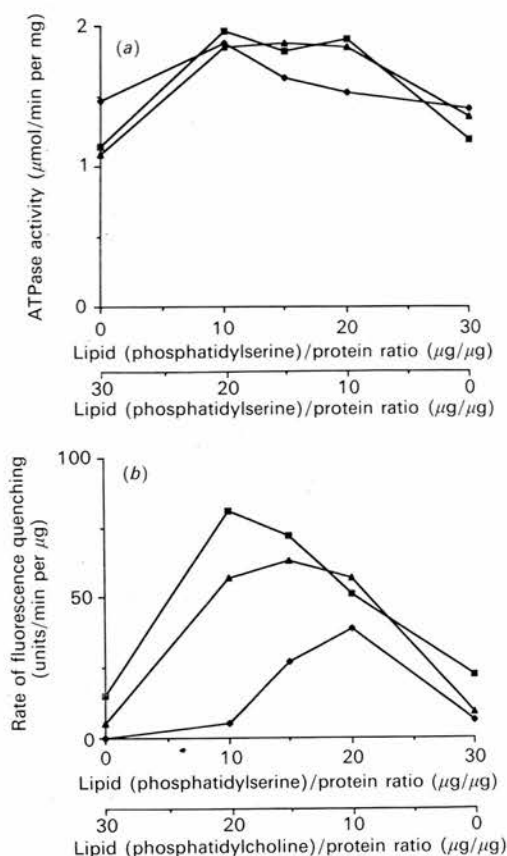


Fig. 5. Dependence of ATPase activity (a) and ACMA fluorescence quenching rate (b) on the phosphatidylcholine/phosphatidylserine ratio

The total phospholipid content was 30 μg/μg of protein and the cholesterol/protein ratio was 0 (◆), 3 (▲) or 6 (■) μg/μg of protein. The valinomycin concentration was 0.4 μg/ml.

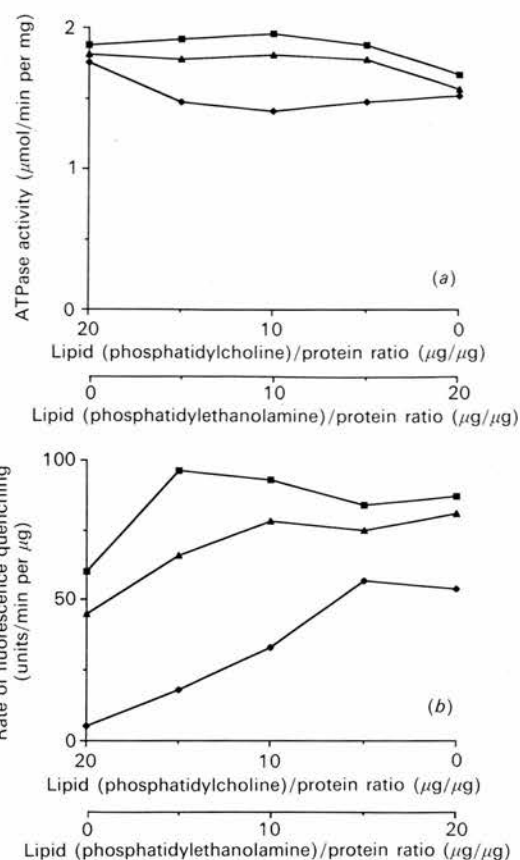


Fig. 6. Effect of replacing phosphatidylcholine by phosphatidylethanolamine

The phosphatidylserine content was constant at 10 μg/μg of protein, and other conditions were as in Fig. 5.

a constant amount of phospholipid, but varied the ratio of phosphatidylcholine to phosphatidylserine, and we examined the effect of incorporating cholesterol into these vesicles. These results are shown in Fig. 5; ATPase activity is relatively insensitive to lipid composition, but is highest with a 2:1 ratio of phosphatidylcholine to phosphatidylserine; the inclusion of cholesterol has little effect. In contrast,  $H^+$  translocation is sharply dependent on the phospholipid ratio and on the cholesterol content of the vesicles. Phosphatidylethanolamine is able to substitute for phosphatidylcholine in reconstitution of the enzyme; Fig. 6 shows the effect of replacing one lipid by the other, the amount of phosphatidylserine and cholesterol being fixed. Independent variation of the amount of phosphatidylserine (results not shown) showed that the optimum was 3 μg/μg of protein. From these results we could define a standard vesicle composition that would give optimal reconstituted ATP-dependent  $H^+$ -translocation activity: this was phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and cholesterol at 9, 9, 3 and 5 μg/μg of protein respectively.

## DISCUSSION

The initial aim of this work was to develop the previously reported procedure of fractionating chromaffin-granule membranes with Triton X-114 to give a product of specific activity comparable with that of the ATPase purified chromatographically. The new procedure can be completed within 2 h,

giving a product that is remarkably stable. It is completely inhibited by bafilomycin  $A_1$  and insensitive to vanadate, so is free of ATPase II (Percy *et al.*, 1985). In the studies by Moriyama & Nelson (1987), reconstitution of  $H^+$ -pumping activity was achieved using endogenous lipids; the ATPase activity of their preparation was slightly higher than that reported here, but the lipid composition of the vesicles was undefined. Our procedure can be modified so that enough endogenous lipid remains to reconstitute ATPase activity, by decreasing the concentration of Triton X-114 to 1.15%, and omitting the second step. ATPase activity is then reconstituted, to a specific activity of about 1.7 μmol/min per mg, on solubilization with octyl glucoside and removal of this detergent by gel filtration; but the vesicles so formed were unable to support a measurable transmembrane pH gradient, so this approach was not pursued. The ATPase pellet has a phospholipid content of < 1 μg/μg of protein, so that the composition of the vesicles was defined by the lipids added.

Vesicular acidification was monitored by the quenching of ACMA fluorescence. A number of observations suggest that the quenching rate is proportional to the rate of  $H^+$  translocation: the proportionality of quenching rate to the protein concentration, its dependence on the addition of valinomycin, its instantaneous reversal by FCCP, and the parallel inhibition by bafilomycin  $A_1$  of fluorescence quenching and ATP hydrolysis.

The observed fractional fluorescence change ( $Q$ ) of self-quenching permeant monoamines such as ACMA is given by:

$$\Delta pH = \log \left( \frac{[ACMA]_{in}}{[ACMA]_{out}} \right) = \log \left( \frac{Q}{1-Q} \right) - \log V$$

